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INTERNATIONAL SYMPOSIUM ON SMALLPOX VACCINE

Proceedings of the 37th Symposium
Organized by the International Association of Biological Standardization
and held at the
Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands
11–13 October 1972

Editors

R. H. Regamey (Geneva) and H. Cohen (Bilthoven)

With 40 Figures and 125 Tables

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EDITORS' PREFACE

The first meeting on microbiological standardization was held in Lyon in 1955. Manufacturers, State controllers and European research workers concerned with the problems of biological standardization came together to try to coordinate their activities and to reach agreement on terminology and requirements. This collaboration rapidly proved to be not only of great interest but also fruitful. Subsequently, the Working Committee became a *Permanent Section* within the framework of the International Association of Microbiological Societies (IAMS).

In 1970, at its Xth Congress in Mexico, the IAMS decided to modify its structure: for instance, a 'Section' could be constituted only by persons proposed by National Societies of Microbiology. Certain former Sections, such as the Permanent Section of Microbiological Standardization, were composed of members elected by the Sections themselves, their members numbering a hundred or more. The General Assembly of the IAMS therefore decided to create '*Commissions*' which, while in conformity with the rules of the IAMS, enjoyed great liberty of organization. In 1971 at its XIIth Congress in Annecy, our 'Section', already transformed into a 'Commission', finally took the name of *International Association of Biological Standardization.*

This Association, as in the past, is composed of persons interested in the problems of the standardization of the control of sera, vaccines and other immunological products, and includes State controllers, manufacturers and independent research workers.

One of the aims of the Association is to promote the development and use of standardized techniques and to support the work of the World Health Organization in the study and application of international biological standards, international biological reference preparations and international biological reference reagents.

The International Association of Biological Standardization organizes three types of scientific meetings:

1. *International congresses* open to all members of the Association and their guests; these usually take place every two years. The topics, which cover a very wide interest, are devoted to the various disciplines of the Association: problems related to research, production and control of immunological products in the field of human and veterinary medicine. Since 1962 (VIIIth International Congress for Microbiological Standardization, Bern) the reports of the *Meetings* have been published in the *Progress in Immunobiological Standardization* (publ. S. Karger).

2. *Symposia* are held on two or more occasions each year and are concerned with single topics; between 30 and 60 specialists in a particular field are selected to attend. If accommodation permits, other members of the Association particularly interested in the subject may attend as observers. The themes are chosen according to their relevance to research, production and control of immunological products. Until now about 40 Symposia have been organized. Beginning with the 12th Symposium (Rabies) the proceedings of these meetings are published in the *Symposia Series in Immunobiological Standardization* (publ. S. Karger).

3. *Meetings of the Committee on Human Diploid Cell Strains.* Meetings of this small international committee are held every year. One of its objectives is to review current developments in the utilization of diploid cell populations in the production of human and veterinary vaccines. The minutes of these meetings are published by our Office.

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The first issue of the *Journal of Biological Standardization* was published at the beginning of 1973.

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Publications of the proceedings of Congresses, Symposia and Meetings are listed at the end of this volume and can be obtained from your booksellers or from our Office (Biostandards, Institute of Hygiene, 1211 Geneva 4, Switzerland).

OPENING ADDRESS

Ladies and Gentlemen,

The Board of Directors of the Rijks Instituut voor de Volksgezondheid – R.I.V. – (National Institute of Public Health) has great pleasure in welcoming you at the opening session of this Conference on Smallpox Vaccine, organized under the auspices of the International Association of Biological Standardization.

A special welcome therefore to the Members of the Council of this Association under the presidency of Dr Perkins.

Smallpox today as a quarantinable disease is rapidly losing its dreaded significance in the world.

Smallpox eradication as programmed and pursued by the World Health Organization seems an objective within reach in the near future. It may be even a matter of only five or ten years. I am therefore very happy to welcome here Dr Henderson and Dr Arita, the chief medical officers in charge of the Smallpox Eradication Unit of the WHO, and Dr Outschoorn, chief medical officer for Biological Standardization of the Geneva headquarters of the same.

We are glad to receive all the participants in this conference in our Institute. We feel we have some connections with smallpox.

In the last 50 years of the history of smallpox vaccination several interesting features having to do with epidemiological, microbiological and virological activities in this field took place in The Netherlands and in the Dutch East Indies.

As early as 1920 Dr Otten developed in the Pasteur Institute in Bandung a 'dried lymph', which could be shipped and stored throughout the entire archipelago of the Dutch East Indies without losing its potency. With the help of this dried vaccine and a systematically continued vaccination programme at the *kampong* level variola was virtually eradicated in that part of the world in the period preceding World War II. This freedom from smallpox was maintained in spite of endemic smallpox in the surrounding states. It is well known that the disease had its 'comeback' in the post-war period of turmoil and disruption, when the vaccination programme was interrupted and no strict surveillance took place. We are happy that today the Indonesian government has succeeded in reorganizing the eradication programme and restoring the former situation.

In the same period before the war smallpox vaccination was severely discredited in The Netherlands. In 1926 the relationship between smallpox vaccination and the disturbing complication of postvaccinal encephalitis was elucidated. Compulsory vaccination of schoolchildren was abolished and investigation committees of the Public Health Council were established to try and clarify this relationship. The quality of the different lymph vaccines was discussed and criticized, but no definite answer could be given.

I see that scientific discussions on the quality-properties of the vaccine, its production and control and the severe complications, including postvaccinal encephalitis are still on your agenda.

The programme for the building of this Institute, which started in 1950, included among other things central laboratories for smallpox vaccine production and testing, for virological diagnostic purposes and for epidemiological services. The scientific staff of these sectors have devoted a great deal of their attention and efforts to the study of several problems concerning smallpox vaccine and smallpox vaccination covered by your Conference. We feel very much honoured that you have chosen our Institute for your meetings and taken the trouble to come to Bilthoven.

I can assure you that the Staff and the Directors of R.I.V. highly appreciate this opportunity to have discussions with so many experts from all parts of the world on the difficult problems related to the use and production of this oldest live vaccine in history, now developing in the direction of the youngest 'historical' vaccine.

We wish you great success at this Conference and hope that its results will give extra support to the world-wide effort of smallpox eradication.

DR J. SPAANDER

Director General of the
Rijks Instituut voor de Volksgezondheid

The Editorial Committee has pleasure in acknowledging the invaluable help of the staff of Professor R. H. Regamey: Mrs B. Duchoud for controlling the texts and Mrs K. Olt for the arduous revision of the bibliographical references. It also extends its thanks for the corrections of the galley-proofs to Miss J. M. Bos, Mrs A. M. P. van Dijk and Miss C. M. M. van der Most, belonging to the staff of the Rijks Instituut voor de Volksgezondheid.

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SESSION I

IMMUNITY AND SMALLPOX ERADICATION

Chairman: Dr A. S. BENENSON (USA)
Secretary: Dr A. A. VOGELZANG (The Netherlands)

International Symposium on Smallpox Vaccine, Bilthoven 1972; Symp. Series immunobiol. Standard, vol. 19, pp. 3–8. Printed in Great Britain

SMALLPOX ERADICATION: THE CRITICAL YEAR AHEAD

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THE FINAL PHASE

Six years ago the World Health Assembly declared the world-wide eradication of smallpox to be one of the major objectives of the Organization. It requested its member states to give the highest possible priority to the provision of necessary assistance in order to accomplish this task in the shortest possible time. It was hoped that the goal might be achieved within a ten-year period.

The response to this request by both endemic and non-endemic countries has been gratifying. In fact, sufficient progress has now been made that it was decided to embark this September on what we have termed the 'final phase' of the programme. Accordingly, intensified surveillance activities are now being undertaken in all remaining endemic areas with the objective, quite simply, of reducing to zero, smallpox incidence throughout the world during the next eighteen months – the next two smallpox seasons.

To some, this might seem an unduly optimistic and overly ambitious goal in the light of current trends in the reported smallpox incidence. Thus far this year over 53 000 cases have been recorded and the eventual total will certainly exceed 65 000 cases. This is twice the number of cases recorded in 1970 and 25 % more than were reported last year. It may also be observed that the 11 000 cases recorded in May this year is the highest monthly total of cases since January 1968. Lastly, it is noted that more cases resulted from importations in 1972 than in any year since the programme began.

However, as we begin this smallpox season, there is, for the first time, some sort of surveillance activity in all endemic areas. During the past year, surveillance activities have begun in the remaining 9 of 14 provinces in Ethiopia, in the southern provinces of Sudan, in western Nepal, in the remaining 3 of 4 provinces in Pakistan, in Botswana and several states of India. While neither the quality nor the intensity of surveillance measures in many areas are yet up to the standard required to interrupt smallpox transmission, steady progress is being made and the gap between what is present and what is required is narrowing.

The increase in reported cases this year reflects this intensified surveillance activity and, as such, is regarded as a favourable sign. The present situation is reminiscent of that in Brazil only three years ago. In Brazil in 1969, after two years of a mass vaccination programme, surveillance activities were begun and progressively extended and improved. The number of reported cases almost doubled as surveillance teams found 20–40 cases for every case reported. Authorities both in the government as well as in other countries expressed

concern and pessimism. Little more than 12 months later, the incidence fell to nil. For more than 18 months now, despite active search by special surveillance teams, no cases have been detected anywhere in the western hemisphere.

DEVELOPMENT OF THE PROGRAMME

The present status of smallpox in the world is perhaps best viewed in the perspective of events of the past six years. In 1967, when the programme began, smallpox was considered to be endemic in 30 countries: in Africa – most countries south of the Sahara; in Asia – Afghanistan, India, Indonesia, Nepal and Pakistan; and in South America, one country – Brazil. Twelve additional countries reported cases believed to have been imported.

As the programme began, it was abundantly apparent that the *sine qua non* for progress was an abundant supply of freeze-dried smallpox vaccine of satisfactory quality. For programmes in the endemic regions it was estimated that approximately 250 million doses would be required. To our dismay, we found that, at best, perhaps 10 to 15 % of the vaccine then in use in the endemic countries was freeze-dried vaccine which met the standards recommended by WHO. With the limited budget available, purchase of this quantity of vaccine was out of the question. Accordingly, it was decided to provide appropriate consultative and material assistance to those laboratories in the endemic countries which appeared potentially to be capable of achieving requisite standards of performance and, for the balance of the requirements, to seek donations from the various countries. The Rijks Institute (Netherlands) generously offered to serve as the WHO International Reference Centre for Smallpox Vaccine and the Connaught Laboratories (Canada) as the Regional Reference Centre for the Americas. Vaccine production laboratories were encouraged to submit regularly, for independent appraisal, batches of vaccine produced. In the beginning, it was felt that few would agree to do this and indeed some were initially reluctant. Surprisingly quickly, however, attitudes changed to the extent that there is now virtually universal co-operation. Since 1967 more than 1300 batches of vaccine have been tested by these two laboratories. In addition, the Reference Laboratories have produced quantities of seed virus for distribution to national producers and quantities of reference vaccine to permit validation of testing procedures; they, with others, have served further to provide consultation to national laboratories and to train their workers; a manual on vaccine production was developed and, finally, a variety of studies were undertaken to evaluate various practical approaches to facilitate production and to assist in solving problems being encountered elsewhere. The contribution of the two Reference Laboratories, as well as that of others, so facilitated the development of vaccine production that now more than two-thirds of the vaccine used in present or recently endemic regions is produced by laboratories in these areas. None produced satisfactory vaccine in 1967.

For the balance of the vaccine required, initially 150 million dose – now less than half of this – donations were sought. Twenty countries have assisted, the largest contributors being the USSR and the USA.

By 1969 more than 95 % of all vaccine in use in the endemic regions was freeze-dried vaccine which conformed to requirements laid down by WHO.

Vaccine needs for the programme were further alleviated when the bifurcated needle, developed by Wyeth Laboratories, was found to be effective in the application of vaccine by the multiple-puncture technique. This device, which we tested for use in this manner in 1967, proved to be a revolutionary addition to the programme. Vaccination technique was vastly simplified and, under field conditions in most countries, savings of 50 % or more in vaccine consumption were realized. The needles are readily sterilized and are

virtually indestructible. Vaccinators in Africa can normally average 500 vaccinations per day and some have regularly vaccinated 1000 to 1500 persons per day over extended periods. The ingenuity of the scientists at Wyeth who developed this instrument must be applauded as well as the generosity of the Wyeth Company, who waived patent charges for manufacturers producing needles exclusively for WHO. With increasing experience and satisfaction with the bifurcated needle, the jet injector, initially employed in South America and Africa, has now been virtually retired from service.

While these problems of vaccine and vaccination were being resolved, WHO co-operated with health authorities to plan smallpox eradication programmes in each of the endemic countries and in many countries particularly vulnerable to the introduction of smallpox owing to their geographical situation. Some programmes began in 1967, but most started in 1968 and 1969. The last, in Ethiopia, began in January 1971. In all, WHO assisted programmes in more than 50 countries.

From the beginning, the strategy of the programme emphasized surveillance as its primary component with mass vaccination a secondary feature. In the past, eradication programmes consisted almost solely of mass vaccination: with the present strategy, surveillance is the keystone.

Within surveillance we encompass broadly those activities related to notification and search for cases as well as the field investigation of outbreaks. By learning all possible about the characteristics of spread of the virus and the people who contract the disease, it is obvious that the vaccination programmes can be directed more efficiently to reach the high-risk groups. In most countries we find that more than 85 % of cases occur among those who have never been vaccinated, and more than 80 % among children less than 15 years old. In many areas a disproportionate number of cases occur among lower socio-economic groups in the cities, and frequently it is individuals belonging to these groups who introduce the disease into rural areas. Accordingly, in most areas, vaccination programmes now place more stress on primary vaccination of children and on the vaccination of poorer city-dwellers.

More important, however, has been the dramatic role of surveillance in rapidly interrupting transmission of smallpox. In fact, we have found that when even a modestly effective surveillance programme has been able to be established throughout a country, transmission has been able to be interrupted within 18–24 months – even in areas where less than half the population has been vaccinated. In contrast, in certain Asian countries without surveillance programmes, transmission has continued despite heroic vaccination programmes which have effectively vaccinated more than 90 % of the population.

The efficacy of surveillance activities in the eradication programme was considerably greater than was originally foreseen. It depends on a number of unique characteristics in the epidemiological behaviour of smallpox. The presence of cases with a visible readily diagnosable rash permits ready identification that the virus is present in the area. While we now know that subclinical infections do occur in partially immune persons, all evidence indicates that these persons play no role in further transmission of the disease and so are of academic rather than practical interest. The disease is sustained by one clinically ill person infecting a second in a continuous chain of transmission. There is no known carrier state and no evidence that the virus may persist in nature and reinfect man. It is readily possible, therefore, to trace the chain of transmission of

smallpox over many weeks to months and so identify previously undetected foci of infection wherever they may have occurred. Surprisingly, the disease spreads far more slowly than most have thought – most individuals with smallpox infect not more than two to five other persons. Outbreaks develop slowly and even after 6–8 weeks can reasonably readily be contained.

These observations have led us to place ever increasing emphasis on surveillance activities although not to the exclusion of systematic vaccination activities. Obviously, if we increase the proportion of immune persons, a partial barrier to transmission is erected which inevitably reduces the chains of transmission that require the attention of surveillance teams. However, it is recognized that systematic vaccination, for the manpower and cost expended, is far less efficient than surveillance.

THE PRESENT STATUS OF SMALLPOX

Since 1967 both the incidence of smallpox and the number of countries reporting cases have decreased significantly. In 1967 the number of cases reported was 131 000. Surveys conducted since 1967 indicate that less than 5% of all cases were then being reported; the actual number of cases that year is thus estimated to have been at least 2·5 million. Despite increasingly complete reporting, smallpox incidence declined each year until 1970, when 33 000 cases – the fewest on record – were reported. This year the number of reported cases will be about 65 000. The completeness of reporting, however, has improved and it is now estimated that at least one third of all cases are notified. The actual number of cases this year is thus estimated to be less than 200 000 in contrast to 2·5 million cases estimated to have occurred in 1967.

The number of countries reporting smallpox decreased from 42 in 1967 to 18 this year. Of the 42 countries reporting smallpox in 1967, 30 were considered to be endemic; the remaining 12 experienced imported cases and outbreaks. At present, continuing transmission is believed limited to seven countries: in Asia – Bangladesh, India, Nepal and Pakistan; in Africa – Botswana, Ethiopia and Sudan.

With the decrease in smallpox incidence, importation of the disease into Europe has become less frequent. During the first two years of the programme, 1967 and 1968, smallpox was imported into Europe on six different occasions, but in the past four years only three importations have occurred – two in 1970 and one in 1972. The last introduction, as you know, occurred in April 1972, when a Yugoslav pilgrim, infected in Iraq, brought the disease back to his own country. No cases have been imported into North America since 1962.

In 1967 the smallpox-endemic countries were considered for operational purposes to fall within four regions: South America, Indonesia, Africa and the mainland of Asia. It was considered unlikely that smallpox would be transmitted between any two of these regions. And, indeed, during the last five years, no such transmission has been detected. Thus, when smallpox transmission is interrupted in one of the regions, it is probable that it will remain free from

smallpox. Two of the four regions, South America and Indonesia, now appear to be smallpox-free.

The last case in South America was detected in Brazil in April 1971. Since this time a continuing active search for possible hidden foci has been in progress in Brazil and in neighbouring countries. None has been found. Brazil, the only country in South America to experience endemic smallpox in the past five years, now has an established surveillance unit in every state and over 4000 reporting posts which report weekly whether or not suspect cases have been detected. Every suspect case is investigated both clinically and in the laboratory by national and/or state surveillance units. It now seems reasonably certain that the western hemisphere, for the first time in 450 years, is free of smallpox.

In Indonesia, the second of the target areas, an eradication programme began in July 1968 in Java and Bali and was subsequently extended to include the outer islands. Surveillance and containment measures were primarily emphasized. During the first three years of the programme between 10000 and 18000 cases were notified annually, but in 1971 the number decreased sharply to 2000. This year, only 34 cases have been reported, all of which occurred in one localized area during January. A national search for cases has been conducted over the succeeding months and special surveillance teams are continuing this activity. No further cases have been detected.

Africa is the third of the target areas. In 1967 smallpox was widely endemic throughout most countries south of the Sahara and during the past six years most have conducted eradication programmes. Except in Botswana, Sudan and Ethiopia, reported smallpox incidence has now decreased to zero. And while health facilities in Africa are limited in number and communications difficult, the existing surveillance programmes in these countries are such that we feel increasingly confident in saying that, in fact, smallpox transmission has been interrupted in all but the three countries noted. Activities in these three countries were reviewed in detail only three weeks ago in a week-long conference in Addis Ababa. Based on present progress and plans it is reasonable to anticipate that transmission in Botswana will be interrupted in a matter of weeks and in Sudan within 6 months. Ethiopia presents a greater challenge, both geographically and logistically. At the same time, it is the last of the eradication programmes to have developed – now being less than two years old. But it is, by far, the most aggressive, imaginative and exciting. In the first year, 1971, over 25000 cases, half the world's total, were reported from Ethiopia. This year the incidence began to decline sharply in March, and already transmission appears to have been interrupted in 6 of the 14 provinces. Eradication of smallpox in Ethiopia – and in Africa itself – is expected early in 1974.

Our fourth and last target area, Asia, is the most problematical. Programmes in Afghanistan and Nepal are well advanced and transmission, if not fully interrupted, appears to be virtually so. However, in the three remaining endemic countries of Bangladesh, India and Pakistan the future is less certain. Ironically, all three have a substantially larger staff of smallpox workers, far better transportation and communication facilities, and better health facilities than in most

countries where transmission has been stopped. The principal problem in India and to a lesser degree Pakistan is that the programmes, until recently, have relied almost completely on mass vaccination in endeavouring to stop transmission. Efforts to improve reporting have only recently been made and the surveillance programme, especially in India, is still far from optimal. Despite levels of vaccination which overall in both countries exceed 80%, transmission persists, as in many areas inadequately supervised surveillance teams ineffectually contain outbreaks. Special efforts are now being made to improve the situation and throughout Pakistan and in many states of India smallpox does appear to be coming under control. Bangladesh has experienced quite a different problem. Having implemented an effective surveillance programme and having interrupted transmission for more than a year, massive outbreaks occurred coincident with the return of refugees from India. Heroic efforts are now being made to again stop transmission.

The effect of measures taken during the past 12 months in the Asian countries will not be able to be fully appraised until several more months of the present smallpox season have elapsed. Presently available data, however, suggest that by June the disease could be confined to not more than four states in India and five districts in Bangladesh.

Advances of this magnitude in Asia, as well as those anticipated in Africa, might seem unduly optimistic, but I can assure you that they are well within reach. The necessary resources are present, adequate staff is available, the techniques are established and the strategy has been demonstrated to be sound. Given a firm commitment on the part of the governments concerned and diligent, dedicated work by the workers in the field, I am confident that the objectives can and will be met in the critical year ahead.

SUMMARY

In the six years during which the intensified global smallpox eradication programme has been in operation, the number of endemic countries has declined from 30 to 7 and the total estimated number of cases from 2 500 000 to 200 000. This year, with the extension of surveillance activities into the remaining endemic areas, the programme enters its final phase. The goal of this phase is to interrupt smallpox transmission in the remaining endemic countries during the course of the next two smallpox seasons.

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SMALLPOX ERADICATION IN WEST AND CENTRAL AFRICA

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INTRODUCTION

In May 1966 the World Health Assembly meeting in Geneva undertook a global commitment to eradicate smallpox in the world by 1976. The United States Government at that time agreed to provide bilateral assistance to 20 countries of West and Central Africa. In contracts with each of the 20 countries, the United States was committed to supply smallpox vaccine, jet injectors and other commodities, while the countries agreed to provide all needed personnel and local expenses. The Center for Disease Control was designated to provide technical assistance and administration of the U.S. portion of the programme.

The 20-country area of West and Central Africa constitutes a single geographic unit bounded by the Atlantic Ocean in the West, the Congo River in the East and the Sahara Desert in the North (Fig. 1). Between 120 million and 130 million people inhabit the area, representing hundreds of tribal groups and a wide spectrum of cultural patterns.

Health structures in the 20-country area reflect the influence of recent colonial powers. In French-speaking areas mobile teams have frequently been used to deliver both curative and preventive health services. In English-speaking areas, the establishment of fixed facilities has traditionally received priority(3).

Smallpox in West and Central Africa has followed long-term cycles with increasing incidence every 4–8 years. A very consistent seasonal pattern was observed in West Africa with a low point in September and October during the rainy season and a peak incidence in March and April, the late dry season. In 1967, the first year of field operations, 14 of the 20 countries reported smallpox, some of the countries reporting the highest smallpox rates in the world (Table I). Retrospective surveys of the prevalence of smallpox pock marks by age-group have indicated the surveillance system was less than 5% efficient(2). Therefore, the 10000 cases reported in 1967 probably indicated an actual total of 200000 cases of smallpox.

PROGRAMME PROCEDURES

Basic operational procedures included mass vaccination, surveillance, epidemiologic investigation, assessment and maintenance. Mass vaccination activities were almost totally carried out by mobile teams who utilized advance publicity to inform villages of the time and day of vaccination. On entering a village, teams would organize the population at one or more collection points and vaccinate all age-groups. During the attack phase over 100 teams worked simultaneously throughout the 20-country area.

Fig. 1. Regional Smallpox Eradication/Measles Control Programme, 20 African countries.

Table I. *Highest smallpox rates in the world*, 1967

Reports 100000 population per year	Country	Reports/ 100000 population per year	Country
68·3	Sierra Leone	13·4	Tanzania
40·2	Guinea	11·7	Indonesia
32·1	Dahomey	10·4	Pakistan
31·7	Niger	9·0	Congo
17·3	Togo	7·8	Nigeria
15·3	India	5·1	Brazil

Surveillance and epidemiologic control procedures were developed to better define the incidence and extent of smallpox, to determine the benefits which were accruing from mass vaccination, and to accelerate control of the disease (1). Four specific methods were utilized. (1) Formal surveillance systems were improved and at the same time informal systems were developed by encouraging other health services, other government personnel, local authority figures, and voluntary agencies to report smallpox cases. (2) Vigorous attempts were made to study all foci of smallpox infection by investigating reports. This included delineating the extent of an outbreak and defining the target area for epidemic control. (3) Outbreak control techniques were used to quickly eliminate

smallpox foci. (4) Rapid communication of smallpox information was encouraged between countries and within countries to facilitate control of outbreaks which crossed administrative boundaries.

Assessment was used in order to provide assurance that the mass campaign was adequately carried out. Fourteen of the 20 countries included assessment as part of routine operations. Assessing a sample of the population 7 days after a campaign allowed programmes to ascertain the percentage of the population covered as well as the vaccination-take rates of those persons vaccinated. Vaccination-take rates in excess of 99 % were repeatedly observed in primary vaccinees. Terminal assessments were done in six countries to determine the immunity status of large areas. These assessments showed that vaccination-scar rates varied from a low of 74 % to a high of over 93 %.

Following the attack phase, maintenance activities have been instituted to achieve two objectives. First, to vaccinate children born since the mobile team last visited a particular area, and secondly, to vaccinate persons of any age who lack a vaccination scar.

RESULTS

Between January 1967 and December 1969 the 20 countries collectively vaccinated an average of 1 million persons every six working days. By December 1969, 100 million persons had been vaccinated against smallpox in the attack phase of the campaign (Fig. 2). At that time many countries completed their attack phase and started maintenance activities. During maintenance activities, most vaccinations were given to children who had been born since the attack phase, therefore the number of vaccinations per month decreased. By the completion of the attack phase, approximately 115 million persons had been vaccinated; over 28 million additional vaccinations have been given during the maintenance phase.

The results in terms of smallpox cases are shown in Fig. 3. No decrease in reported smallpox was demonstrated in West Africa in 1967. Reported incidence in 1968, while lower than the previous year, was within the expected range as compared to previous years. The expected seasonal increase, however, was not observed in 1969 and 19 of the 20 countries became smallpox-free by October of 1969. The last cases of smallpox were reported in May of 1970. A surveillance system has continued and has been sufficiently sensitive to detect seven cases of human monkeypox in the last 2 years, yet no smallpox has been observed in West Africa for $2\frac{1}{2}$ years. West and Central Africa cannot be considered safe, however, until the entire African continent has become smallpox free.

In addition to the elimination of smallpox in the 20-country area, another result of the West African campaign has been the further development of surveillance and epidemiologic control as a potent tool for smallpox eradication. Epidemiologic control methods were advocated in England during the nineteenth century when it was clear that mass vaccination methods were not eliminating smallpox (4). As a practical concept, however, surveillance and epidemiologic control developed slowly and found their greatest application in non-endemic countries attempting to detect and eliminate importations.

In 1967 an approach of 'selective epidemiological control' was introduced in

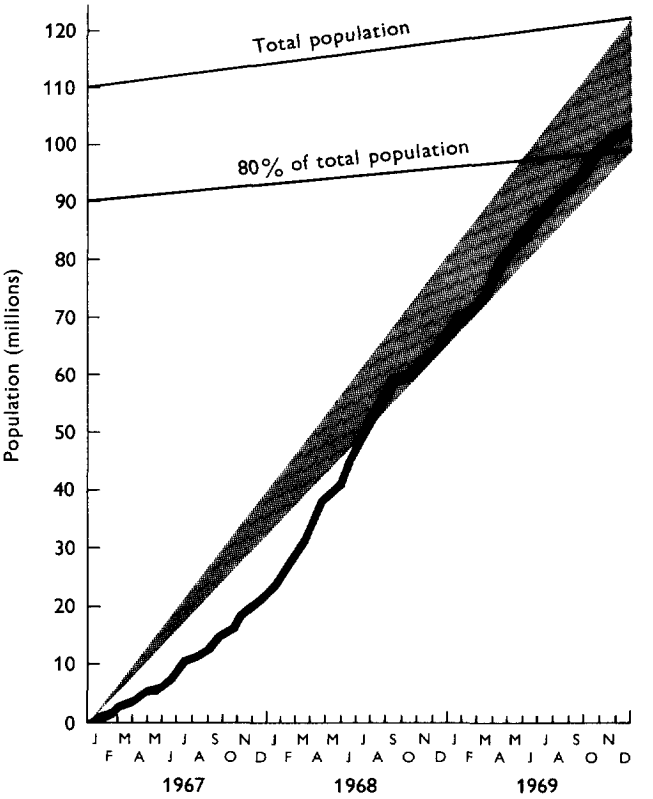

Fig. 2. Cumulative smallpox vaccinations as related to the estimated total population. West and Central Africa Smallpox Eradication/Measles Control Programme area.

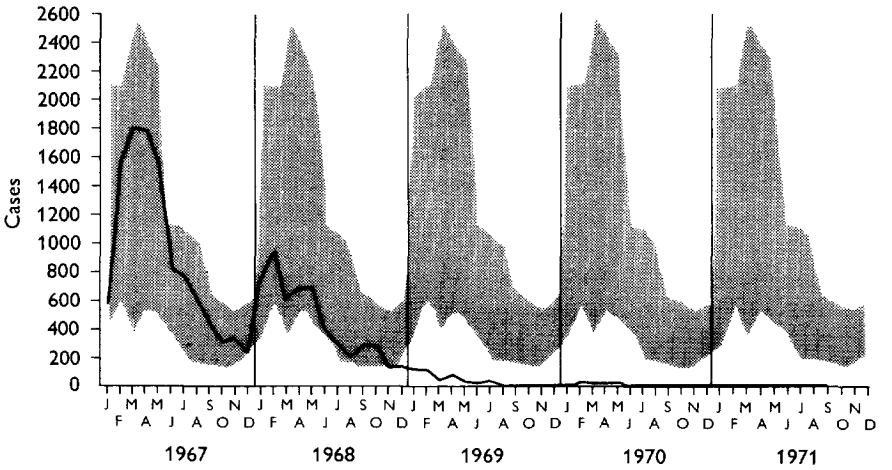

Fig. 3. Africa, West and Central: smallpox incidence, 1967–71. The stippled area represents the range between the highest and lowest incidence reported during the 5-year period 1962–6.

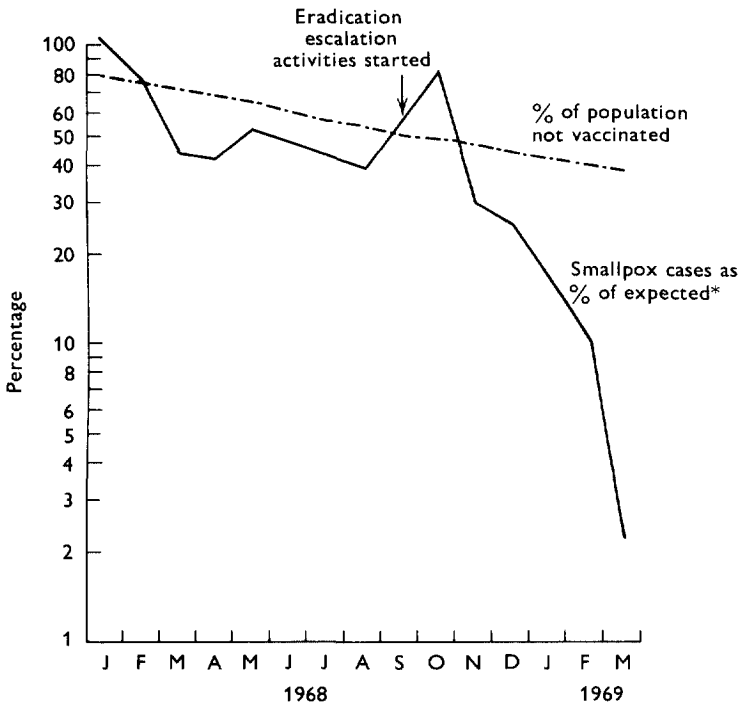

Fig. 4. Percentage of population not vaccinated in the Smallpox Eradication Programme area compared with the ratio (%) of reported smallpox cases to the expected smallpox cases. * 1960–7 monthly average.

certain areas of West Africa. The objective was to emphasize detection of outbreaks and to break chains of smallpox transmission rather than rely on mass vaccination. The strategy was applied widely in West Africa in September of 1968. September was selected in order to capitalize on the low point of smallpox when the fewest chains of transmission would exist. Results are shown in Fig. 4. The decline in smallpox susceptibles as the result of mass vaccination is shown and compared to the decline in smallpox cases expressed as the ratio of observed to expected cases. Although reported smallpox cases increased immediately after the introduction of selective epidemiologic control techniques, the rate of smallpox decline thereafter was much more rapid than observed with mass vaccination alone. The use of this technique in endemic areas is now widely accepted and is based on the fact that smallpox tends to be a focal disease with relatively slow spread.

The cost of smallpox eradication in West and Central Africa is shown in Table II. The 5-year cost per capita was 13·8 cents with a cost breakdown as follows: programme administration, commodity purchase and the movement of teams to villages cost, 9·7 cents; local indirect costs and local salaries cost, 1·8 cents per person; cost of smallpox vaccine, 1·6 cents; cost of using jet injectors, 0·7 cents per vaccination.

Table II. *Per capita costs of smallpox eradication in West and Central Africa*

	$
Commodities, administrative, and delivery costs	0·097
Local salaries and local indirect costs (estimated)	0·018
Smallpox vaccine	0·016
Jet injectors (purchase and spare parts)	0·007
Total costs per person	0·138

DISCUSSION

Several issues have been clarified by the West African experiences. First, the decision of 20 countries to provide a co-ordinated regional attack on smallpox was not only commendable but was essential in achieving smallpox eradication. Secondly, high quality programmes with good coverage and take rates were possible even in areas devoid of other forms of medical infrastructure. Thirdly, cost figures indicate that a major expense of the campaign involved the process of getting a team to a village. This suggests that efficient immunization programmes of the future should administer the maximum number of antigens to each person, thereby reducing the frequency of team visits to each village. Multiple antigen immunization programmes should be a natural extension of the Smallpox Eradication Programme. Fourthly, the use of selective epidemiologic control techniques accelerated the decline of smallpox and these techniques are now considered as important as mass vaccination activities. Fifthly, the absence of smallpox cases in West Africa for 2½ years could be disrupted by importations from East Africa. Continuing maintenance vaccination programmes and vigilant surveillance-control capabilities will be required at least until the entire continent becomes smallpox-free.

SUMMARY

A regional smallpox eradication programme was initiated in 1966 with field vaccination activities beginning in January 1967. Over 100 million persons were vaccinated by December 1969 and smallpox transmission ceased in May 1970. Maintenance vaccination programmes are continuing in order to vaccinate newborns and persons without a vaccination scar. Surveillance-control activities must remain effective at least until the remainder of Africa becomes smallpox-free.

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VACCINATION FACTORS CRITICAL FOR ERADICATION OF SMALLPOX

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One hundred and seventy-five years ago Edward Jenner reported the effectiveness of the mild cowpox infection in protecting against lethal smallpox. The significance of this work was immediately appreciated. Within four years, James Bryce wrote:

> Dr Jenner has thus acted his part; it remains for the other members of society to act theirs; he has shown how important advantages may be obtained; it is theirs to carry this plan into execution by co-operating, both by example and by precept, to render general the practices of inoculation for cowpox: the reward being no less than exterminating one of the most loathsome and fatal diseases which mankind are liable – The smallpox. [5]

Slowly over the years, the incidence of smallpox declined as vaccination campaigns were carried out. Since World War II, Europe and the United States and Canada have been free of the disease except for small outbreaks after importations by travellers from relatively unvaccinated areas. But it is only within the past few years that smallpox has been eliminated from the Western Hemisphere, and countries of Africa which had the highest endemic rate of disease have been rendered free of this 'the most loathsome and fatal disease to which mankind are liable'. The World Health Organization programme is entering the final phase of its programme with the goal of achieving global eradication within the next eighteen months.

Final success will not be attributable to any dramatic new discoveries since the day of Jenner, but to careful identification of the errors in implementing the techniques laid down long ago, and a belated international determination to accomplish what Bryce had foreseen in 1802.

PROBLEMS OF PUBLIC ACCEPTANCE

The greatest obstacle to vaccination has always been the reluctance of the public to accept the procedure. Antivaccinationists have been with us since the days of Jenner, motivated by fear and distrust of the new. The superstitious feared that cows' heads would emerge from their bodies, while religious zealots were convinced that interference with the ways of the gods would bring horrible retribution. Superstition has indeed been a formidable opponent.

Unfortunately, the procedure for vaccination has not been entirely innocuous.

Vaccinia is associated with definite clinical symptoms, which are more intense when more virus is inserted over larger skin areas. Because the vaccines in use were inconsistent in their infectivity, greater and greater amounts of trauma were used in the vaccination process, with elaborate scarifiers, multiple insertion sites, cruciate incisions, etc.; these increased the likelihood of a take but these takes were more likely to be associated with significant illness. A vicious cycle developed. It became necessary to achieve immunity with a single isolated contact since a second contact with a subject would be problematical. The use of traumatic devices such as the rotary lancet on multiple sites did, in fact, tend to ensure immunity in those to whom it was applied but police action was not infrequently required to protect the vaccinator from the wrath of the mob or to force the subjects to submit. The inevitable consequence was avoidance of the vaccinator by large segments of the population, leaving, even in theoretically vaccinated communities, large susceptible blocks of people.

Not only was the trauma of vaccination increased, the strains of vaccine virus were selected which produced larger and more consistent reactions. Strains were passed from man to rabbit to calf to man to increase its 'virulence'. The use of these virulent strains in higher and higher concentration applied by more rigorous methods intensified the severity of reaction and consequently public resistance.

While much vaccination reluctance was based on the illness associated with the normal vaccinal reaction, as smallpox incidence declined greater emphasis has been placed on the adverse complications or accidents of vaccination. Tetanus still could be seen very recently in remote areas where manure was applied to rotary lancet vaccination sites hoping to abort the 'take' with its anticipated severe illness. The complications of vaccinia itself, such as post-vaccinal encephalitis, eczema vaccinatum, etc., will be discussed later in this conference; however, the concern over these very rare complications have assumed such importance now that the fear of the complications exceeds the fear of smallpox, even in the face of smallpox itself.

The solution to the great problem involved in inducing the public to accept vaccination has depended on intensive health education, and revision of our methods to minimize illness, based on our newer information.

THE PROBLEM OF INADEQUATE VACCINE POTENCY

Vaccination depends on an adequate concentration of live virus in the vaccine to initiate infection. Too often in the past, vaccine was released by the producing laboratories with no attempt to determine the viral content. The more developed countries have required for years that vaccine be tested by rabbit scarification or other methods to assure an adequate viral content. The development of the pock-counting technique on the chorioallantoic membrane, and then tissue-culture plaque counting, has made viral titration relatively simple. The WHO has set standards which call for a titre of 10^8 pock-forming units per ml. The current smallpox eradication programme is based on vaccines with adequate

potency, and our host laboratory here in Utrecht serves as the reference laboratory to assure that the vaccines in use in that campaign meet the requirements.

Unfortunately, after release from the producing laboratory, the virus often deteriorated and led to vaccinations without effecting immunization(3). Some of us have lived through the dismaying experience of a smallpox epidemic among vaccinated people. Jenner himself had appreciated that vaccinia virus could be held for long periods of time when dried(10). Otten demonstrated the practicalities of a dried vaccine in achieving control of the disease in the Dutch East Indies(14). Collier(6) developed the present methodology which makes available to us freeze-dried vaccines which can be immersed in boiling water for an hour or held for four weeks at 37 °C without significant loss in infectivity. The present general availability of freeze-dried vaccine assures that we can vaccinate in any part of the earth with potent material.

Even these highly effective vaccines can be ruined by bad vaccinating techniques. Too often, reports are received detailing the use of vaccinostyles or other implements for vaccination which are sterilized between patients by heat or by a chemical agent. The implement was then dipped into the vaccine bottle for the next vaccination, and the heat or chemical ruined an excellent vaccine. Thus, while all of the many vaccination techniques used over the years can protect against smallpox, it was evident that new techniques were necessary which would immunize the subject with reasonable reliability and minimal inconvenience.

VACCINATION TECHNIQUES

The multiplicity of traumatic techniques have been replaced by mild techniques applied to single insertion sites. While the scratch method has been preferred in Europe, success depends on the skill of the vaccinator(4). The multiple pressure method popularized by Leake in 1927(12) has been the preferred technique in the United States; this is not equally effective in all hands. This is well demonstrated by a study in which two preventive medicine physicians revaccinated the same subjects, one on each arm, using vaccine from the same bottle by the multiple pressure technique (Table I). It is evident that there was a great difference in the frequency with which they evoked major reactions, despite the use by both of what appeared to be proper technique. With more experience, comparable success rates were achieved.

The problem of technique has been resolved by two approaches, one rather complicated and the other very simple. The jet-injector was developed to inject a measured amount of vaccine intradermally; this called for an injectable vaccine free of bacterial contamination. While the jet-injector needs maintenance personnel with reasonable mechanical ability, it makes available to the vaccinator a device with which many people can be rapidly immunized with no danger of harming the vaccine or transmitting infections such as serum hepatitis(1). The West African campaign was based on jet-injector vaccination.

A much simpler and very cheap device is the bifurcated needle, which can be made available in sufficient numbers that sterilization between subjects should

Table I. *Influence of operator on vaccination result (same vaccine applied to both arms at same time)*

Series	Number revaccinated	Major reactions (%) Right arm (Dr A)	Left arm (Dr B)	Both
1	40	30	52	55
2	23	9	65	70
3	21	38	67	57
4, 5	34	68	62	76
Total	118	38	60	67

Table II. *Comparison of multiple puncture and multiple pressure vaccination (same vaccine applied to both arms at same time)*

	Number of punctures 5	10	15
Number vaccinated	149	144	150
	% of total major reactions detected		
Multiple punctures	81·4 %	93·7 %	93·8 %
Multiple pressure (15–20 pressures)	99·0 %	92·6 %	88·5 %
Index: puncture: pressure	0·82	1·01	1·06

not be needed. The vaccination technique was simplified to that of multiple puncture rather than pressure. Comparisons with multiple pressure indicated its superiority in creating infection. In this small study (Table II) groups of military recruits were vaccinated on one arm by the multiple pressure method, using 15–20 strokes of the bifurcated needle; the other arm was vaccinated with the same vaccine using 5, 10 or 15 punctures into as small an area of skin as possible. The reactions were examined 1 week later and it is evident that the multiple-puncture method surpasses the multiple pressure method in the more rigorous test of revaccination.

STRAIN SELECTION

Over the past several years efforts have been under way to select strains for vaccination which were effective in immunizing and yet produced less morbidity. Polak(**15**) in Holland and Marennikova *et al.*(**13**) in the USSR have been particularly involved in selecting standard vaccinal strains which would produce minimal morbidity. A new approach has been that of prevaccination procedure

to avoid the serious complications of vaccination and to reduce or eliminate the morbidity. Attenuated strains such as the CV-1 have been studied by Kempe(**11**) in the USA and by van der Noordaa *et al.*(**16**) in Holland. Others have studied the use of killed or non-living vaccines, particularly in Europe(**7**). Further amelioration of adverse symptoms is suggested by Espmark who showed a reduction in morbidity when the virus dose was reduced(**8**).

TARGET POPULATION

Given a vaccine of adequate potency and effective techniques acceptable to the public, the problem is still not solved until the critical population has been protected. Disease transmission ceases when herd immunity is sufficiently high. The WHO Expert Committee in 1964(**17**) recommended that the goal of the vaccination campaign should be 100% of the population because the earlier objective of 80% of the population had been misapplied. That goal could be achieved by vaccinating those who were most easily accessible, leaving unvaccinated and unsought those who were more difficult to reach; it was among these that smallpox often persisted and from whom it could spread to other groups who might be unprotected. It was beautifully shown by the experience in West Africa reported by Foege *et al.*(**9**) that smallpox control is achieved when that segment of the population in which smallpox exists is rendered non-susceptible; this is the logical target of an eradication programme and not the total population.

We have reached the point now that James Bryce was concerned about in 1802 when he wrote:

> I must here, however, observe that it is not the prevention of smallpox in a country, for a few years or perhaps a century, that ought to be regarded sufficient... If it should then unfortunately so happen that the advantages resulting from cowpox are forgotten, or undervalued...then the smallpox may again be imported from some remote corner where the influence of cowpox was unknown...and hold a course among mankind nearly as terrific as that described by authors who relate the ravages of this dreadful disease... Measures might be contrived not only for rendering vaccine inoculation general, but also for continuing it with unremitting diligence throughout future ages.[**5**]

We look forward to the eradication of this disease from the populations of the earth, but until the last pox virus has been removed from the last deep freeze and destroyed, and we are assured of no virus lurking somewhere in a primate or other animal reservoir, we need to remain armed with the tools for its control if it should be 'imported from some corner', i.e. potent vaccine with proper methods of application by people who know how to use it. Studies have been initiated so that we may be better prepared in the future if Bryce's concern should materialize(**2**). Dr Galasso will give a preliminary report of the early results.

SUMMARY

Despite the availability of smallpox for a long time, eradication could not be envisioned until all segments of the public were prepared to accept vaccination. This has been achieved by development of less traumatic vaccination techniques and reliably potent vaccines. With these in hand, it has been possible to reach the populations in which the disease persisted with steady progress toward achieving eradication from all known foci.

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ANTIGENIC DIFFERENTIATION OF MONKEYPOX*

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Monkeypox virus as it was discovered by Von Magnus *et al.*(**2**) belongs to the vaccinia subgroup of poxviruses. It can be distinguished from variola virus by a haemorrhagic pock character, higher ceiling temperature (39 °C instead of 38·3 °C), higher chick embryo lethality (the D 4 value of the virus being about 1·5 or 2 logs lower), serial transmission in rabbit skin and RK 13 cells, and by a higher haemagglutinin titre in CAM.

A white mutant can be obtained from classical monkeypox virus isolates. Such white mutants differ from the parent strain by its white pock character only. Otherwise they resemble monkeypox virus.

We obtained from cynomolgus healthy monkey kidney cells two poxvirus isolates which differed from monkeypox as was first noted by Dr Marennikova. These isolates resembled variola virus and were called 'white poxvirus' to avoid any prejudice regarding their classification(**3**). A similar variola-like virus was isolated from kidney cells of a chimpanzee by Marennikova *et al.*(**1**).

Isolates of monkeypox, its white mutant, variola and the three white poxvirus isolates from healthy monkeys were compared by means of immunodiffusion against antivaccinia serum in agarose. We applied a technique which is used in our laboratory for routine tests on Australia antigen that is associated with serum hepatitis. Fresh CAM extracts and an extremely potent antivaccinia serum (6801) were used. The neutralization of Elstree vaccinia virus by this antiserum presented in the graph shows that the antibody titre of the serum corresponding with the 50% point of pock reduction was $10^{5\cdot63}$.

Variola extracts when tested along with monkeypox Copenhagen against this antivaccinia serum showed a double spur formation indicating a reaction of partial identity. The spur antigen was called VA-antigen. The spurs were less distinct with other less-potent antivaccinia sera, but could be demonstrated after tenfold concentration of such a serum by means of lyphogel. Monkeypox virus could not stimulate antibodies against the variola specific spur antigen. This negative result, however, requires further investigation. An analogon of the VA-determinant in monkeypox might be less antigenic and could have been overlooked due to technical circumstances.

Extracts of the three white poxvirus isolates from healthy monkeys showed reactions of complete identity with variola extracts and reactions of partial

* An extension of this paper will be published elsewhere.

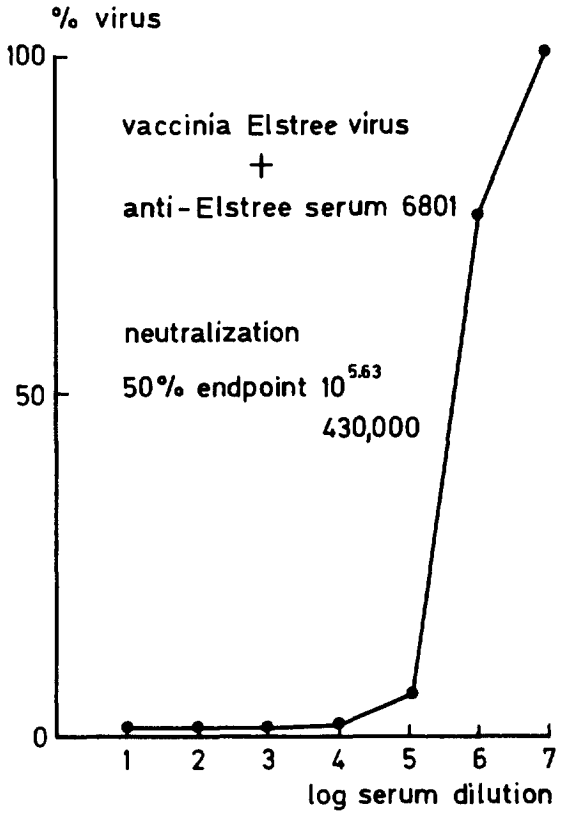

Fig. 1. Neutralization of vaccinia virus (pock counts) by antivaccinia serum 6801.

identity with monkeypox extracts. The white poxvirus isolates could thus not be distinguished from variola virus.

The absence of VA-reactivity in monkeypox extracts could also be proved by absorption experiments. Antivaccinia serum 6801 was absorbed with monkeypox Copenhagen. The absorbed serum reacted with variola, not with monkeypox virus. The exhaustion of the serum as to antibodies for common antigens required repeated saturation with the virus antigen, a process that resulted into a three-fold dilution. It was shown, however, that dilution as such was not responsible for the antibody exhaustion.

A number of pox viruses serologically related to the vaccinia sub-group were tested for VA-antigen. All 9 isolates from 7 variola outbreaks, each of the 3 white poxvirus isolates and 3 vaccinia strains were VA-antigen-positive, whereas 10 monkeypox isolates from 6 outbreaks in monkeys and 3 in humans were VA-antigen negative. White mutants from monkeypox (Copenhagen and Rotterdam isolates respectively) were also VA-negative. VA-reactivity could therefore

Table I. *VA-antigen in poxviruses according to gel-precipitation along with monkeypox Copenhagen isolate against antivaccinia serum* 6801

Poxvirus isolates	Number		
	Tested	Positive	Negative
Variola major, 6 outbreaks	8	8	0
'White' poxvirus, 2 episodes	3	3	0
Variola minor, 1 outbreak	1	1	0
Vaccinia, 3 strains	3	3	0
Monkeypox, 9 outbreaks	10	0	10
Monkeypox white mutant	2	0	2

be used to obtain a differentiation between variola and vaccinia isolates on one side and monkeypox on the other (Table I).

Although the antigenic structure of vaccinia comprises the VA-antigen component, the presence of an additional specific antigen in vaccinia could not be excluded. The protective effect of smallpox vaccination against monkeypox has been experimentally proved in monkeys. So far the results of our analysis were consistent with this procedure of immunization.

SUMMARY

A variola-specific antigen appeared to be present in all 9 isolates from 7 variola outbreaks and each of 3 vaccinia virus strains tested. It was absent in 10 monkeypox isolates derived from 6 outbreaks in monkeys and 2 in humans.

The antigenic differentiation between monkeypox and vaccinia viruses is consistent with the protective effect of smallpox vaccination against monkeypox.

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Discussion

HOCHSTEIN-MINTZEL (Germany) Is it possible to induce experimentally fatal infection with monkeypox virus in monkeys? Can monkeypox challenge serve as a model for the testing of immunogenicity of smallpox vaccine?

GISPEN (The Netherlands) Monkeys are susceptible and can be experimentally infected with monkeypox virus. Local and general reactions may occur. Lethality of the infection

depends on the route of inoculation. Most lethal are aerogenic infections, especially natural infections such as we had in an outbreak in the Zoo of Rotterdam. Testing of immunogenicity of smallpox vaccine requires a model for natural infection. Such a model is difficult to set up with monkeys and would be very expensive.

HARBOE (Norway) What is the result of testing anti-monkeypox serum against monkeypox antigen and vaccinia antigen?

GISPEN (The Netherlands) As monkeypox antiserum does not contain antibodies against V-antigen the result was a reaction of complete identity. However, when vaccinia extract was tested along with monkeypox extract against antivaccinia serum, the presence of precipitable V-antigen in vaccinia could be demonstrated.

BENENSON (USA) Can you tell us the source of the tissue culture cells from which the variola-like virus was isolated?

GISPEN (The Netherlands) These were Cynomolgus monkey kidney tissue culture cells used for routine diagnostic work. There was no known possible contact of these animals with pox viruses. These monkeys had come from Malaysia, and had been in quarantine here for 52 days before the kidneys were taken.

BENENSON (USA) Dr Marennikova, can you give us some information on the chimpanzee from which the variola-like virus was isolated?

MARENNIKOVA (USSR) This was a chimpanzee in the Congo; he was easy to capture and so may have been convalescent from disease. There was monkeypox in the area, but there had not been human variola for many months.

ESPMARK (Sweden) Is the antigenicity of monkeypox distinguishable from variola and vaccinia? Is there any evidence that monkeypox is in fact smallpox?

GISPEN (The Netherlands) The differences between monkeypox and variola viruses are genetically stable. There is no indication of change in one or the other direction. Monkeypox in humans did not cause secondary cases so far. There is no reason to fear that it will interfere with smallpox eradication.

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THE EFFECT OF SMALLPOX VACCINATION IN THE UNITED KINGDOM

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The first person claiming to have vaccinated subjects in the United Kingdom is Benjamin Jesty, who used material taken directly from the udder of a cow to vaccinate his wife and two sons, Robert and Benjamin, in 1774, and at a later unknown date he vaccinated Mary Brown. The gravestones recording these events are in Worth Matravers church, Dorset, and there appears to be no earlier records of a successful vaccination.

It appears that the work of Jesty was disputed by Jenner largely because he felt that Dr Pearson was behind it. It was Dr Pearson of St George's Hospital and Dr Woodville who set up a vaccination practice in Golden Square which became known as the Vaccine-Pock Institution. Jenner expected to be offered the directorship but was offered only a post as 'extra consulting physician', which incensed him. This started a battle between the Jesty and Jenner families which went as far as petitions to Parliament, but all failed to obtain the desired response. In spite of this, however, it is generally believed that Jenner was the first person to place vaccination on a scientific footing.

The Vaccination Act of 1853 made primary vaccination of infants compulsory (see CMO report for 1919(**2**)). Although subsequent Acts modified this legislation, they were not finally repealed until the inception of the National Health Service Act of 1946 in the year of 1948.

During the years between 1855 and the turn of the nineteenth century the number of deaths due to smallpox was the highest in 1871 at 23000 deaths and the lowest in 1890 at 16 deaths(**3**). The average number of deaths was of the order of 1000–2000. At the beginning of the twentieth century the number of deaths dropped markedly to as low as 2 or 3 per annum and the highest peak was 53 in 1928. Since 1935 there have been very few deaths due to smallpox, the highest year being in 1962 when there were 26 deaths. In these 37 years there have been 25 years in which there have been no deaths and there were only four occasions on which the number of deaths reached double figures. The record looks even more impressive if one takes the last 13 years, because apart from the one year of 1962, which was our last small epidemic, there have been no years in which there have been any deaths due to smallpox.

Taking the incidence of cases in recent years, the record is equally impressive. Since 1911 there have been few years in which the number of cases of smallpox

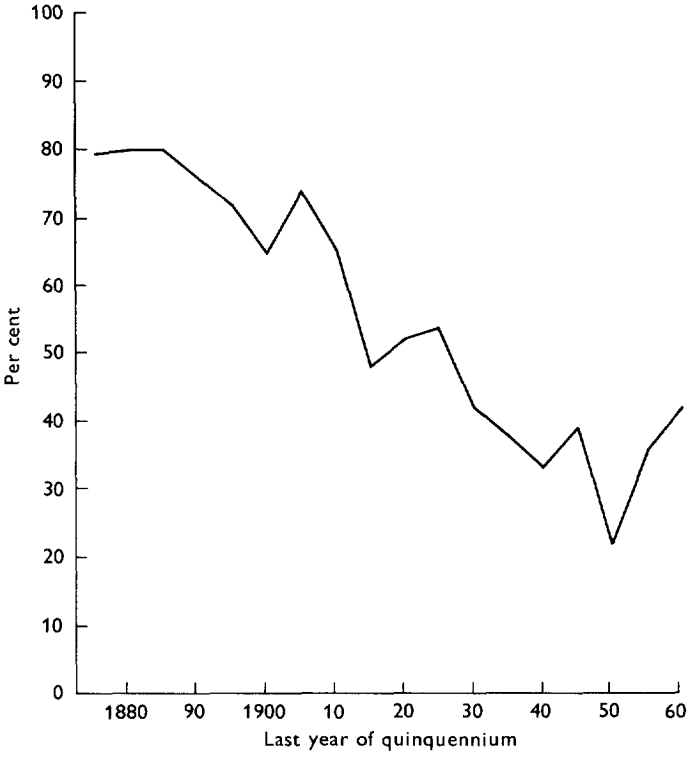

Fig. 1

have reached over 100. Indeed, apart from the years between 1922 and 1934, when there was a period in which there were outbreaks of variola minor reaching a peak of nearly 15000 cases, there have been few cases of variola major. Apart from 1952 and 1966, when variola minor cases boosted the figures, we have had very little smallpox in the United Kingdom.

Fig. 1 shows the acceptance rates (the proportion of infants born in any one year who are vaccinated per 100 total births) between 1875 and 1960. It is interesting to note that in 1874 and for the following 20 years our acceptance rate was over 80%. By 1894, however, the number of cases and deaths had fallen dramatically and this may have had an effect upon the acceptance rate, which fell to 61% by 1898.

It is important also to take into consideration the success of vaccination and the part played by the Vaccination Acts. In the 1870s and early 1880s, although our acceptance rate was over 80% at that time, vaccination was from arm to arm and there is no indication of the success of such a procedure. Between 1882 and 1898 the rate fell to 61%, but following the Vaccination Act of 1898 the rate rose to nearly 76%. After 1905, however, the rate fell progressively to 20% in 1948 at the time of the repeal of the Act.

The variola minor outbreak of 1952 again reminded the population of the

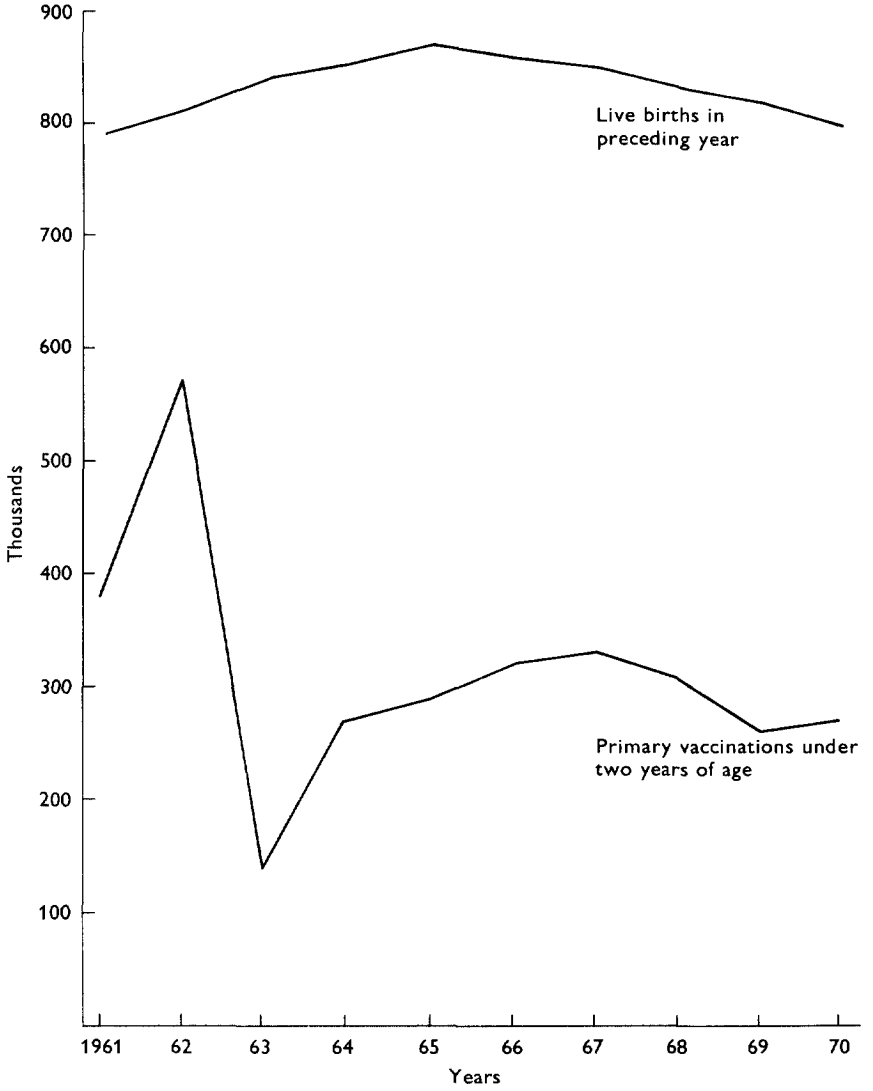

Fig. 2

need to be protected against the disease and by 1959 the acceptance rate had crept up from 23 in 1950 to 45 in 1959. Since that time we have had a gradual fall in our acceptance rate, apart from 1962 (see Fig. 2) when again we had variola minor, until today when we have less than 40% of children vaccinated. This was the situation up to the time when the Department of Health and Social Security recommended that smallpox should be no longer part of the routine immunization procedure and the figures of children being vaccinated must now be extremely small.

Dr E. T. Conybeare, when he was at the Department of Health in 1964, reviewed the complications and deaths from smallpox vaccination between 1951 and 1960(1), from which it was clear that deaths were occurring as a result of vaccination. These data, together with the belief that the smallpox eradication programme of the World Health Organization would inevitably decrease the risk of an imported case of smallpox into the United Kingdom, led to the recent policy of abandoning vaccination as a routine measure.

This symposium will reveal the developments that have been made in the production of bacteriologically sterile smallpox vaccine made and tested by modern techniques. Hopefully, the use of such vaccines may decrease the risk of smallpox vaccination complications to such an extent that it may be used with safety. It is possible that the World Health Organization programme, though being highly successful, may not result in eradication. If there remain pockets of epidemics of smallpox and if the populations from these areas enter the United Kingdom, then I feel that we may have to re-evaluate our policy with respect to smallpox vaccination.

The figures that supported the policy of stopping vaccination as a routine measure were taken from a population which, in the main, was protected against the disease, but these figures will no longer be relevant if in ten years time we have built up a majority without any immunity against smallpox and if subsequently we have smallpox entering the community, especially if the case remains undiagnosed for any length of time.

If we do have to return to vaccination as a routine measure then we shall surely use a bacteriologically sterile product shown to be safe and effective in man. This conference will be a landmark in history of progress in the production of such a vaccine.

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Discussion

EDSALL (England) Your remarks suggest that bacterial contamination such as it is seen in current smallpox vaccine is responsible for some of the major complications. Can you verify this inference or expand on it?

PERKINS (England) We hope that information on this question will become available.

DEKKING (The Netherlands) Would you not like to go to ultimate consequences and state here that even without world-wide eradication infant vaccination need not to be reinstituted in the British Isles because they are well protected by their excellent Public Health Services?

PERKINS (England) Provided that an early diagnosis is made of an imported case of smallpox then 'ring vaccination' should work. We should not forget, however, that the imported case that started the outbreak in 1962 was not diagnosed for several days. The outbreak of 1962 might have been very much worse, had we had a non-immune population as we shall have in the United Kingdom in ten years time.

SESSION II

PRODUCTION OF SMALLPOX VACCINE

Chairman: Dr P. FENJE (Canada)
Secretary: Dr E. C. BEUVERY (The Netherlands)

International Symposium on Smallpox Vaccine, Bilthoven 1972; Symp. Series immunobiol. Standard, vol. 19, pp. 33–38. Printed in Great Britain

ENHANCED YIELD OF SMALLPOX VACCINE BY PURIFIED SEED VIRUS

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Smallpox vaccine is still mainly produced on animal skin. The method of vaccine production has not been significantly changed during the past century. All the other viral vaccines for human use are produced at present either in eggs or tissue cultures. During the last decades a number of factors have been discovered which can increase or decrease the multiplication of viruses both *in vivo* and *in vitro*. The yield of virus in vaccine production might, therefore, be increased by optimizing the growth conditions of the virus (for references see Valle(3)). Humoral specific antibodies and cell-bound immunity are the best known among the factors controlling virus infections. Viral interference phenomena and interferon are other examples of the viral inhibitory mechanisms.

The possible role of interferon in the smallpox vaccine production on calf skin was studied in this investigation. Wheelock(5) showed that the dermal crusts of human vaccinia virus vaccinations contained interferon and Armstrong *et al.*(1) observed that the vesicle fluid of these vaccinations also contained interferon. Therefore the aim of this study was to examine whether the seed virus, used for calf inoculation, contains interferon and whether this affects the vaccine production.

MATERIAL AND METHODS

Vaccinia virus The Finnish strain of vaccinia virus was used (Valle(3)). It has been continuously passed on calf skin for the last 30 years at least.

Production of smallpox vaccine A glycerinated calf-lymph type of smallpox vaccine was produced in our laboratory. The method fulfils the requirements for smallpox vaccine given by the WHO(6). One volume of the harvested pulp was homogenized primarily in 4 volumes of 0·004 M McIlvaine's buffer. Before further processing this bulk material was tested for potency by chorioallantoic membrane method (Valle(3)). The final bulk used for smallpox vaccinations in our country as well as the seed virus used for calf inoculation contained 40 % glycerol buffered by 0·004 M McIlvaine's buffer.

Arcton treatment For purification of bulk material 10 % of Arcton (trifluorotrichloroethane) was added. After homogenization for 1 min a centrifugation for 5 min at 365*g* was performed. The virus containing supernatant was harvested by decantation.

Purified seed virus The purified seed virus used for calf inoculation was prepared from the supernatant of the Arcton treatment. It was centrifuged for 60 min at 15000*g*. The sediment obtained was resuspended in the original volume.

Table I. *Interferon content of unpurified and purified seed virus*

Virus preparation	Interferon units/ml
Unpurified	100
Unpurified	< 3*
pH 2 dialysed	100
Supernatant†	100

* Assayed in human amnion cells.
† Centrifuged for 60 min at 15000*g*.

Table II. *Effect of purification on the seed virus preparation*

Treatment of seed virus	Vaccinia virus log pfu/ml	Protein content (g/l)*	Interferon (units/ml)
None	8·63	6·5	100
Arcton	8·73	3·2	100
Supernatant†	4·33	2·7	100
Sediment‡	8·74	0·6	< 3

* Measured by the Lowry method.
† Arcton treated preparation centrifuged for 60 min at 15000*g*.
‡ Resuspended in the original volume of 40% glycerol in McIlvaine's buffer.
pfu = plaque forming units.

Protein content The protein content was measured by the normal Biuret method and by the Lowry modification using the Folin–Ciocalteau phenol reagent (Lowry *et al.* (**2**)).

Assay of interferon The interferon activity was measured by the 50% vesicular stomatitis virus plaque inhibition method (Valle & Cantell (**4**)) in secondary cultures of calf kidney cells.

RESULTS

The interferon activity of homogenized pulps originating from different calves was 30–100 interferon units per ml. The activity was measured in secondary cultures of calf kidney cells. The sera from the same calves taken before virus inoculation showed no interferon activity and the sera taken at the time of virus harvest contained 3 or < 3 interferon units per ml.

The interferon activity was characterized as shown in Table I. To exclude the interference of live vaccinia virus with vesicular stomatitis virus, which was used as challenge virus in the assay of interferon activity, vaccinia virus was inactivated by incubating the samples for 30 min at 56 °C. There was no interferon activity detectable by human amnion (U-line) cell cultures. The interferon activity was resistant to an overnight pH 2 dialysis. Vaccinia virus is known to

Table III. *Yield of calf lymph vaccine by unpurified and purified seed virus*

Experiment no.*	Yield per calf (pfu × 10^{10})	
	Unpurified	Purified
1	69·9	126·4
2	72·0	148·4
3	76·4	164·4
4	23·6	71·2
5	20·0	69·2
6	93·2	129·6
7	52·0	70·8
8	96·0	96·4
9	73·2	190·8
Mean	64·0 ± 9·07	118·5 ± 14·84

* In each experiment two calves were inoculated either by the unpurified or the purified seed virus.

pfu = plaque forming units.

be sedimented by centrifugation for 60 min at 15000*g*. However, all the interferon activity stayed in the supernatant.

The effectivity of some virus purification procedures, used in the production of smallpox vaccine, was studied next in respect to the potency, protein content and interferon activity (Table II). The virus preparation used in this experiment was made by homogenizing 1 g of pulp in 11·1 ml of 0·004 M McIlvaine's buffer. It was observed that the Arcton treatment decreased the protein content by about 50%, but loss of virus or reduction in interferon activity could not be observed. A similar low-speed centrifugation without the addition of Arcton decreased the protein content to 3·7 g/l. When the supernatant from the Arcton treatment was further purified by centrifugation for 60 min at 15000*g* the virus was sedimented. Practically all the virus was in the sediment which contained only about 10% of the original amount of protein. Essentially the same results were obtained when the protein contents were determined by the Lowry and the Biuret methods. All interferon activity remained in the supernatant.

A study was performed where unpurified vaccinia virus and a high-speed centrifuged virus of the same potency were used as seed virus preparations. Nine calves were inoculated with both seed virus lots respectively. The yields of virus by these calves were compared (Table III). The calves inoculated with the unpurified seed virus gave an average yield of $64{\cdot}0 \times 10^{10} \pm 9{\cdot}07 \times 10^{10}$ pkfu per calf. The calves inoculated with the purified seed virus yielded $118{\cdot}5 \times 10^{10} \pm 14{\cdot}84 \times 10^{10}$ pkfu per calf respectively. This means an 87·5% increase ($P = 0{\cdot}005$) in the yield of vaccine. The mean weight of the pulps was 131·0 ± 7·8 g per calf by the unpurified seed virus and 135·8 ± 5·9 g per calf by the purified seed virus respectively.

DISCUSSION

Similarly to the findings of Wheelock(**5**), the calf crusts of vaccinia infection also contained interferon activity. This activity was shown to meet the main criteria for interferon. The activity reduced the plaque formation of vesicular stomatitis virus. It was resistant to pH 2 dialysis and to heat-inactivation for 30 min at 56 °C. It showed species specificity as it was active in calf cells but not in human cells. The activity was not sedimented by centrifugation for 60 min at 15000*g*. Therefore it is evident that the inhibitory factor which was observed in the unpurified seed virus was interferon. However, unpurified smallpox vaccine may also contain other inhibitory factors.

The finding that the pulp contained much more interferon activity than the calf serum at the time of harvest is in good agreement with the findings of Armstrong *et al.*(**1**) from human vaccinations. The activity found in the calf sera were, however, too low to allow any firm conclusions.

The results obtained showed clearly the advantage of the use of a purified seed virus. The simple treatment of the seed virus increased the vaccine yields highly significantly. It would be interesting to study whether similar interference phenomena may affect the yields of other viral vaccines *in vivo* or *in vitro*. The presence of calf interferon in the smallpox vaccine should not influence the human vaccinations because of the species specificity of interferons. However, if human cells are used for vaccine production it is not impossible that interferon in the vaccine may affect the vaccination result.

SUMMARY

An unpurified or partially purified seed virus is generally used for the production of smallpox vaccine. The unpurified seed virus lots for calf inoculation were shown to contain 30–100 interferon units per ml. The interferon activity could not be separated from the virus by low-speed centrifugation or Arcton treatment. However, when vaccinia virus was sedimented by centrifugation for 60 min at 15000*g* the interferon activity remained in the supernatant.

The effect of the interferon activity on smallpox vaccine production was studied by inoculating calves by an unpurified or a purified seed virus of the same potency. In a series of nine pairs of calves the average yield of virus was increased from $64{\cdot}0 \times 10^{10}$ to $118{\cdot}5 \times 10^{10}$ pkfu per calf by the use of the purified virus. This means an 87·5% increase in the yield of the vaccine.

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Discussion

NETTER (France) (1) Were the unpurified and purified seed viruses used in different calves? (2) Were the clinical symptoms (local or general) in the calves different when purified or unpurified seed virus was used?

VALLE (Finland) (1) Yes, different calves were used. (2) No, clear differences could not be seen. The duration of the fever and the maximal fever were the same in both groups.

DICK (UK) How do you know that it was the removal of the interferon that was the important factor in increasing the potency of your seed virus? You said there may be other inhibitors; they could be more important. What you can conclude is that purified seed virus is more effective in vaccine production.

VALLE (Finland) As I told you I was aware of the possibility that some other inhibitors may have been removed when the seed virus was purified. The interferon activity which was shown to be present in the unpurified seed virus and which had been removed from the purified seed virus can however explain the favourable effect obtained. According to the present knowledge on the replication of vaccinia virus in calves, interferon seems to be the most probable factor causing the decreased yield of virus obtained by the unpurified seed virus.

HOCHSTEIN-MINTZEL (BRD) How can interferon activity be measured in an unpurified suspension of vaccinia virus from calf skin (protein content 6·59/l, $\sim 10^9$ pfu/ml).

VALLE (Finland) All the samples tested for interferon activity were inactivated by incubating them for 30 min at 56 °C. Interferon is known to be resistant to such a treatment but vaccinia virus is inactivated. Therefore the possible interference between live vaccinia virus and the vesicular stomatitis virus, which was used as the challenge virus, was inhibited.

EHRENGUT (BRD) It is known that the virus content is much lower in the scabs of patients with variolois than with variola. In view of Wheelock's paper, do you think that interferon induction is still working many years after a smallpox vaccination; in other words, could the lower infectivity of the varioloid patients be due to such mechanism?

VALLE (Finland) This is an interesting question which has not been studied according to my knowledge. I know that this type of phenomenon is going to be studied and they may give us a new parameter to evaluate the effectivity of different vaccine strains.

POLAK (The Netherlands) Is the higher total yield in pock forming units related to a higher concentration per gram of pulp or to a higher pulp harvest?

VALLE (Finland) As given in the text, the mean weight obtained per calf in the two groups was practically the same. Therefore the increase in the yield depended on a higher concentration of virus per gram of pulp.

LEMOINE (Belgium) I understood that Dr Valle worked only with bovine seed. I suppose that there would be no effect on the purification of rabbit seed if it is used for the inoculation of calves?

VALLE (Finland) I have not worked on rabbit seed, but according to the data given here by Dr Marennikova there is interferon activity in the rabbit pulp too. If this pulp is used to inoculate calves its interferon content should not interfere with the replication of vaccinia virus because of the species specificity of interferons.

MARENNIKOVA (USSR) Am I right that your 'purified' virus (Table III) is the sediment without interferon?

VALLE (Finland) Yes.

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ISOLATION OF VACCINIA VIRUS FROM DERMOVACCINE

A COMPARATIVE STUDY OF MODERN PREPARATIVE METHODS FOR VACCINE PRODUCTION

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The isolation of intact virus particles from accompanying proteins which are present in smallpox vaccine is the main requirement of modern vaccine production. The antigenic and allergenic character of these substances can result in sensitization and repeated application can cause local and general complications. There are also some persons who are accidentally sensitized and the corresponding antigen may be present in vaccine.

Such events have been reported in connexion with measles vaccine(**3**). Bonin(**2**) recently reported one case of anaphylactic shock and several allergic skin reactions after the injection of vaccinia antigen, and according to these authors these reactions are caused by accompanying calf serum. We have also observed this type of reaction in some persons.

These problems also arise with smallpox vaccine. For this reason it is necessary to examine the conventional production method and if possible to develop new techniques to obtain a vaccine which is free from toxic substances and allergenic proteins.

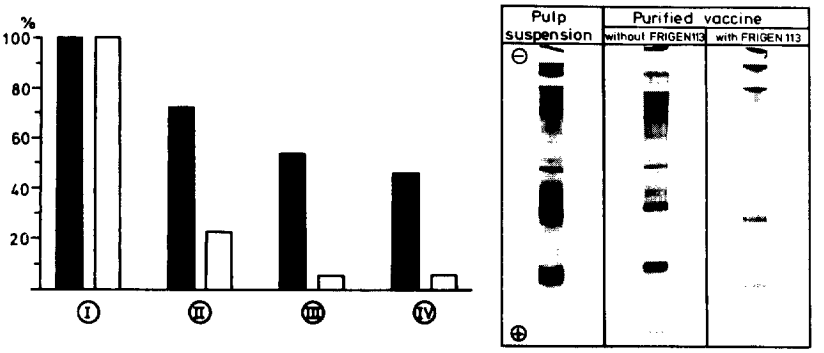
Naturally the main feature of production must be high yield of virus with minimum protein content and the production must be inexpensive.

With this aim in mind we considered the applicability of column chromatography on agarose gels and controlled-pore-glass as well as gradient centrifugation for production. We therefore experimented with these methods and compared the results with the conventional differential centrifugation.

METHODS AND RESULTS

For the investigation we used suspensions of untreated sheep dermal vaccine (1 : 10, w/v, in McIlvaine buffer, pH 7·2) (series A) and suspensions treated with 10% Frigen 113 (1,2,2-trifluorotrichloroethane/fluorocarbon; Farbwerke Hoechst AG) (series B). The coarse particles were removed by centrifugation at 1000*g* for 10 min. The supernatants are starting materials for chromatography and zonal centrifugation.

To control the results we estimated viral activity on chorioallantoic membrane (**5**) and the proteins were estimated according to the methods of Lowry(**11**) and Goa(**9**). Electrophoresis on cellulose acetate membranes (CAM), immunoelectrophoresis (**5**) and disk electrophoresis (**6, 13**) were used as indicators of purification.



		Viral activity		Protein content			Viral loss during individual procedure
	n	% recovery	% loss	g/100 ml	%	$R_{eff.}$	(%)
(I) Pulp suspension	27	100	—	2·52	100	1	27·7
(II) Supernatant after	27	72·3	27·7	0·57	22·6	3·2	
Frigen 113 treatment		(100)			(100)	(1)	
and 1000 *g* for 10 min							25·6
(III) Pellet after	60	53·8	46·2	0·13	5·2	10·3	
10000 *g* for 60 min		(74·4)			(22·8)	(3·3)	
(resuspended)							15·9
(IV) After freeze-	60	45·2	54·8	0·13	5·2	8·7	
drying		(62·6)			(22·8)	(2·7)	

Fig. 1. Purification of sheep dermal smallpox vaccine with Frigen 113 treatment and subsequent differential centrifugation. Disk electrophoresis shows the striking difference between the untreated vaccine and the vaccine treated with Frigen 113. $R_{eff.}$ = effective purification grade. ■, Viral activity (%), □, Protein content (%).

The effective grade of purification was designated as the $R_{eff.}$ value, which means the percentage viral activity divided by the percentage protein content. The starting-material has 100 % virus and 100 % protein, so it has an $R_{eff.}$ value of 1.

Absorption curves were registered at 254 nm with 3 mm cuvettes.

I. *Differential centrifugation* We have been using this conventional production method for freeze-dried vaccine since 1967. The pulp is suspended in McIlvaine buffer (1:9, w/v) and homogenized. After Frigen 113 treatment (10 %) the suspension is centrifuged at 1000*g* for 10 min for the removal of coarse particles. The supernatant is centrifuged at 10000*g* for 60 min. After this the viral pellet is resuspended in McIlvaine buffer containing 5 % peptone-sorbitol and freeze-dried. In this method the total viral loss amount is 45 % while the protein content is reduced to 5 % (Fig. 1).

Without Frigen 113 treatment but purified by differential centrifugation the protein content of final vaccine is 40–50 % more (0·23 g/100 ml as against 0·13 g/100 ml).

Disk electrophoresis shows about 30 bands of protein in unpurified as well as in vaccine treated with Frigen 113. More than ten bands are always distinct. The conventional purified preparation still contains trace amounts of these bands, which are clearly detectable in disk electrophoresis.

Chromatographic material and treatment of sample	*n*	Virus fraction: Viral activity (%)	Virus fraction: Protein content (%)	$R_{eff.}$
Sepharose 6B (M_{lim} 4 × 10⁶)				
Without Frigen 113	7	40·6	42·9	1·0
With Frigen 113	8	13·8	14·4	1·0
Sepharose 4B (M_{lim} 20 × 10⁶)				
Without Frigen 113	6	34·4	28·6	1·2
With Frigen 113	9	20·2	9·5	2·1
Sepharose 2B (M_{lim} 40 × 10⁶)				
Without Frigen 113	11	29·0	22·3	1·3
With Frigen 113	10	14·6	3·7	3·9
Bio-gel A-150 m (M_{lim} 150 × 10⁶)				
Without Frigen 113	8	22·6	13·8	1·6
With Frigen 113	8	17·6	8·2	2·1
CP-Glass: Corning, CPG 10–2000 (M_{lim} 12 × 10⁶)				
Without Frigen 113	8	19·2	8·6	2·2
With Frigen 113	7	15·5	3·7	4·2

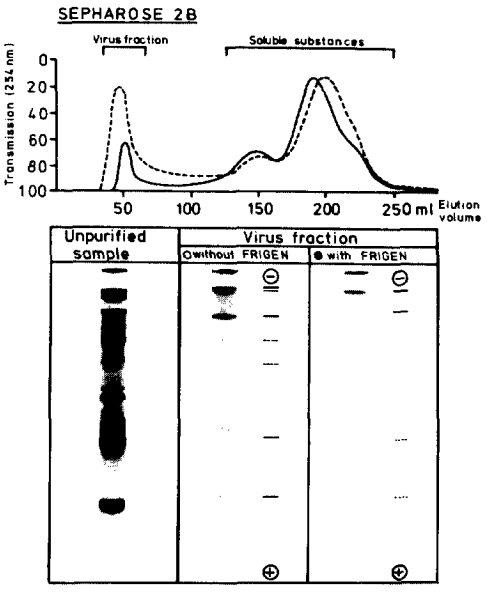


Fig. 2. Isolation of vaccinia virus from dermovaccine by column chromatography. – –, Sample without Frigen 113 treatment. ——, Sample with Frigen 113 treatment.

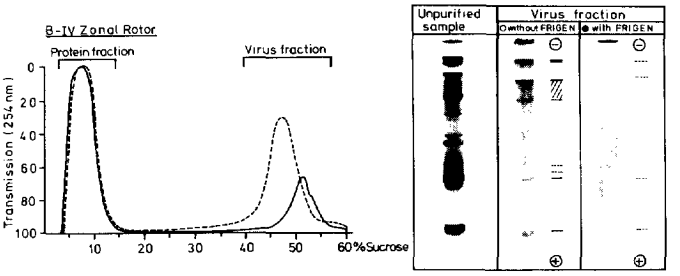
CAM electrophoresis shows 5–6 fractions and immunoelectrophoresis 4–7 precipitating lines in unpurified vaccines. Since disk electrophoresis is most sensitive only the pictures of this method are represented in this paper.

II. *Column chromatography* Hedström & Lindberg(**10**) as well as Stickl and his co-workers(**14**) indicated the applicability of gel filtration for the purification of vaccine. In our experiments we used (Sepharose 6B, 4B, 2B, Bio-gel A-150 m and controlled-pore-glass Corning CPG 10–2000. About 170 ml of chromatographic material was used for columns and samples of 5–6 % of this volume, i.e. about 10 ml, were applied on the top of the columns and eluated with 0·004 M McIlvaine buffer (pH 7·2).

The principle of this technique is that substances with molecular weight higher than the exclusion limit of the chromatographic material are eluated first, while low-molecular-substances which are retained by pores are separated.

Since the vaccinia virus has very high molecular weight it is always eluated at first with the exclusion volume. In most of the experiments viral loss was up to 80–85 %. From Table II it is evident that agarose gels with an exclusion limit of more than 20 millions molecular weight and CPG 10–2000 can reduce most of the accompanying soluble substances. But it is necessary to use pulp pre-treated with Frigen 113. Fluorocarbon-treated samples always gives smaller and distinct virus peak. Disk electrophoresis also shows better separation (Fig. 2).

III. *Zonal centrifugation* The separation of biological material in density gradient with zonal centrifugation was first tried by Brakke(**4**). The principle of this separation is based on the sedimentation velocity of a substance in density gradient, which depends upon



		Position of fractions in sucrose density gradient						Virus fraction		
		Protein fraction			Virus fraction			Viral activity	Protein content	
	n	Start	Peak	End	Start	Peak	End	(%)	(%)	$R_{eff.}$
Sample without Frigen 113 treatment	13	3·8	8·0 (g 1·030)	14·3	39·6	47·3 (g 1·215)	54·3	73·7	22·2	3·3
Sample with Frigen 113 treatment	13	3·1	7·5 (g 1·028)	13·8	45·9	51·8 (g 1·239)	57·0	59·7	9·0	6·6

Fig. 3. Isolation of vaccinia virus from dermovaccine by zonal centrifugation in sucrose density gradient (B-IV Zonal rotor). – –, Sample without Frigen 113 treatment, ——, Sample with Frigen 113 treatment. The position of fractions is given in percentage sucrose.

the size, density and shape of the particle. Anderson in 1963 indicated the applicability of this method for the production of vaccine(1).

But up to this time this method has not been used for smallpox-vaccine production. Dostal(7) indicated in his paper on swinging-bucket rotors that a zonal rotor can be successfully used for the production. For this purpose we used a B–IV zonal rotor in an L-4 Beckman ultracentrifuge. Following were the conditions for the experiments: overlay, McIlvaine buffer (0·004 M, pH 7·2), 50 ml; sample, 50 ml; gradient, 10–60 % (w/w) sucrose in buffer, 1350 ml, linear; cushion, 60 % (w/w) sucrose in buffer, 275 ml; centrifugation, 30000 rev/min for 75 min; $g_{min.}$: 17568; $g_{max.}$: 51115.

In the case of a crude suspension without any treatment the optical density of the virus fraction is high and in the majority of experiments the peak was uniform. The size and base of the peak indicate the presence of non-viral particles.

After treatment with Frigen 113 the virus peak becomes sharp and distinct – similar to the peaks in column chromatography. The peak appears to consist of 2–3 closely associated fractions and the position is shifted to higher density level. In these experiments the protein is reduced to 9 %, with viral loss of 40 % (Fig. 3). In a few of our experiments we applied this technique to continuous-flow zonal rotors (B-XVI and JCF-Z, Beckman instruments).

In the high cylindrical rotor B-XVI with small sedimentation distance there is a danger of overloading of gradient zones and an aggregation of virus in viral fraction. This can be avoided by using a flat cylindrical rotor (like the JCF-Z) with longer sedimentation distance and lower speed.

DISCUSSION

The unpurified vaccine is a mixture of cellular particles as well as soluble substances in which vaccinia virus is present as a specific component. Most of the soluble substances correspond to serum protein spectrum of the host, and amongst these proteins gammaglobulin and albumin are immunologically noticeable. Most of these substances can be eliminated, as we have shown, far better in column chromatography and zonal centrifugation than in commonly used differential centrifugation.

In all these experiments the important factor is the Frigen 113 treatment. The prepurification with fluorocarbon gives a homogeneous suspension clear and free of coarse particle, but it still contains all soluble proteins. It appears that Frigen 113 does not react with soluble proteins, but it interacts with surface structures of non-viral particles which adsorbed proteins. These particles are sedimented (**8**). Probably an affinity to cell and bacterial membranes containing lipid appears to be the cause. Furthermore it appears that the virion is attacked by Frigen 113. This can be easily observed in zonal centrifugation, where the sedimentation behaviour of the virion is significantly changed after Frigen 113 treatment. It may be that adsorbed proteins on the envelope of the virion are dispersed and some components from the outer surface of the virus particle are separated (**12**). This can be the cause of the increased instability of the pretreated viral suspension.

Certain problems arise in applying the new techniques for production.

The column chromatography has its limitations because of high viral loss: the chromatographic material probably retaining the virus. Beyond this the isolated virus particles are exposed to chemical and physical effects. A further disadvantage is the relatively rapid contamination of chromatographic material. In this respect CP glass is superior to gels since cleaning, regeneration and sterilization can be done very easily and rapidly.

In zonal centrifugation the viral loss is comparable to the conventional purification method. There are technical difficulties; one of them is sterilization. By using a stepwise gradient (without a pump system) and a new type of rotor with uncomplicated filling and continuous-flow devices these problems can be minimized.

On the basis of our findings the following points should be noted:

(*a*) The conventional purification method which makes use of Frigen 113 and differential centrifugation is suitable for vaccines which are applied by epidermal scarification.

(*b*) For the production of a vaccine to be used for intracutaneous, subcutaneous and intramuscular application or jet injection, the zonal centrifugation in density gradient is a method of choice and this method must be further developed. This is evident from the comparison of different results (Fig. 4).

(*c*) The homogeneous and primary sterile tissue culture vaccine and egg vaccine offer considerable relief in this field. It must be tested whether column chromatography gives better results for these vaccines, but we cannot imagine

	Conventional purification (Diff. Centrifugal)		Column chromatography Agarose		Column chromatography CP-Glass		Zonal centrifugation B-IV Rotor		Continuous flow zonal centrifugation B-XVI Rotor		Continuous flow zonal centrifugation JCF-Z Rotor	
	% Virus	% Protein	% Virus	% Protein	% Virus	% Protein	% Virus	% Protein	% Virus	% Protein	% Virus	% Protein
Sample (after FRIGEN 113 treatment)	100	100	100	100	100	100	100	100	100	100	100	100
Purified virus preparation	74,4	22,8	17,5	7,1	15,5	3,7	59,7	9,0	44,2	2,4	85,2	3,8

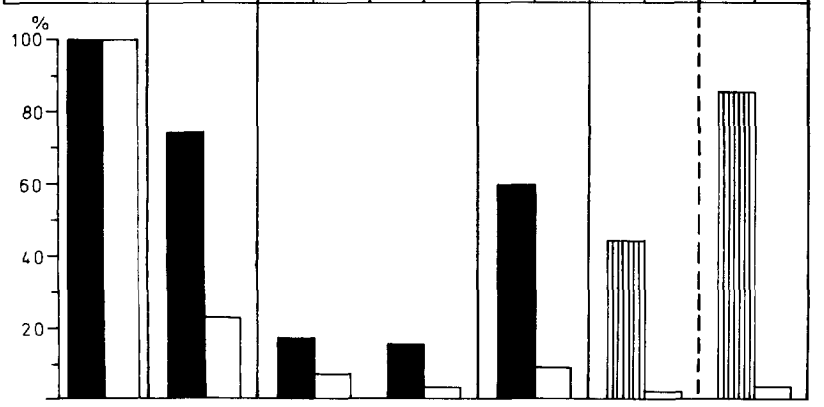


Fig. 4. Summary of results of different purification methods. ■/▥, Viral activity (%). □, Protein content (%).

that this method is applicable for large-scale production. For successful purification the prerequisite is the elimination of non-viral particles with fluorocarbon. It is worth further investigation to find out a solvent which will improve this step to prevent reaction with viral particles. A method of sterile filtration of highly purified virus preparations must be developed because there is not a single purification method which completely separates bacteria from the vaccinia virus.

By developing and applying modern techniques for vaccine production it will be possible to prevent unspecific allergic events.

SUMMARY

The results of column chromatography on agarose gels, controlled-pore-glass and zonal centrifugation in sucrose density gradient are compared with that of differential centrifugation, which is commonly used for smallpox vaccine production.

Column chromatography and zonal centrifugation eliminate most of unspecific soluble substances which are present in dermovaccine.

A prerequisite for isolation of virus is the treatment of pulp with fluorocarbon (Frigen 113), which diminishes host-cell particles.

Since in column chromatography there is a great viral loss the zonal centrifugation is recommended for production of highly purified vaccine.

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Discussion

NETTER (France) (1) Can this highly purified vaccine be used for a long time? (2) Is this vaccine used for vaccination of humans and what are the post-vaccinal reactions?

RICHTER (BRD) (1) In our laboratory we have stored this vaccine at −20 °C for 5 years without appreciable loss. At 4 °C one can keep the stabilized vaccine for 2 years. (2) Regarding post-vaccinal incidents, we do not have any data with relation to purified vaccine. However, we do not believe that the cause of post-vaccinal complications is related to unspecific accompanying substances.

HOCHSTEIN-MINTZEL (BRD) In our experience density-gradient centrifugation gives much lower recovery (~ 20 %) of total infectivity.

ESPMARK (Sweden). Was the purified preparation as stable in the freezer as ordinary smallpox vaccine? Or did you add protective substances like serum, sugars or other chemicals to obtain a satisfactory stability in the frozen state?

RICHTER (BRD). The purified freeze-dried smallpox vaccine which is obtained after Frigen 113 treatment and subsequent differential centrifugation is stabilized with 5 % peptone-sorbitol. The stability of this preparation is very good. For the last 5 years we have been using this method for routine production. We believe that the isolated virus preparations by column chromatography and zonal centrifugation can also be stabilized in the same manner.

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DEVELOPMENT AND QUALITY CONTROL OF FREEZE-DRIED SMALLPOX VACCINE IN INDIA

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In India smallpox vaccine is manufactured on animal skin. Essentially the production schedule is similar to what has been described in the WHO Manual no. SA/68.3, 'Methodology of freeze-dried smallpox vaccine'. The minor deviations are mostly due to local factors. We use buffalo calf, preferably female, aged below 1½ years and weighing between 80 and 100 kg. There is no organized livestock breeding in the country and the animals are procured from heterogeneous source on contractual basis. Mixing of healthy and diseased calves, therefore, is a hazard. Close scrutiny during selection and quarantine by a competent veterinary service is enforced to weed out the diseased from the healthy. The calves on admission receive prophylactic vaccination against Black Water and haemorrhagic septicaemia. While incidence of tuberculosis in the livestock population is scanty, foot-and-mouth disease virus infection amongst the calves in the past totally disrupted our vaccine production.

VACCINE PRODUCTION

The animals on admission are given a thorough soap bath and the skin is liberally sprayed with Cetavlon 1%. The quarantine period is 14 days. During the quarantine they are fed on dry hay, pelleted food, and salt. Total change over to pelleted diet has failed. Animals are reluctant to change their food habits and when forced there is a greater mortality during incubation – death being due to gastroenteritis.

No local antiseptic is applied once the animal has been prepared for vaccination. Routinely scrupulous hygienic care is enforced during incubation, which consists of quick removal of excreta from the floor and platform and continuous flushing of the floor with running water. In one institute we have tried Cetrimide 1% spray on the vaccinated site during incubation with limited success. A rich harvest and minimum bacterial contamination can be achieved when the animals receive parenterally 2 g and 8000 units of streptomycin and procaine penicillin respectively on the evening before the application of the seed virus and the injections are continued till the day of harvesting (this method is being used in one institute). Criss-cross scarification is carried out in stages. About 3 in

of skin area adjacent to the vertebral column on each side of the trunk is not vaccinated. The harvesting is done at 96 h. The average pulp yield is 300 g/calf. Random bacteriological monitoring of the vaccinated area is a routine and starts after the first day's incubation till harvesting. Routine autopsy is carried out to ensure freedom from any gross pathological conditions.

During homogenization the buffer is phenolysed (phenol 0·5%). Pooling of the suspension is not favoured and also is not required. The four different projects manufacturing freeze-dried smallpox vaccine are using Edward Shelf Dryers which can accommodate 4000 ± 100 ampoules of specific dimensions per run. The quality of the final product was variable at different centres earlier. This variability was traced to the freeze-drying schedules. It was noticed that the schedule was mostly tailored to suit local conditions and conveniences. Our experience shows that besides the initial pock count of seed virus pool, pre-freezing of the suspension appears to be of utmost importance in achieving a potent and stable vaccine. The minimum time is 10 h. Heating is applied soon after the vacuum has attained 100 μm and is regulated according to the capacity of the condenser to take the vapour. Maintenance of a maximum differential between the product and the condenser temperature is essential. During secondary drying agitation and change of P_2O_5 ensure minimum moisture content. Vaccine manufactured under such conditions should not only fulfil the usual requirements but should pass the stringent boiling test.

SEED VIRUS

In the past the manufacturing units were employing different seed strains for the manufacture of vaccine. For the last two years the seed strain is uniform and the second generation of the same seed lot has been supplied to all the units. High potency, purity, stability, immunogenicity and reactogenicity of the final product is intimately linked with the seed quality. The ancestry of the seed system is uncertain. Although not documented, the original seed seems to have been collected from a case of arm-to-arm vaccination, in preference to two other strains, and presumably of cowpox virus. In the year 1933 this seed lot was mixed with one received from the UK (Lister Institute) and the resultant seed was considered superior.

With the introduction of the chorionic–allantoic membrane technique for potency, heterogeneity in the morphology of the seed virus was confirmed. There are two distinct populations – one thermostable at 100 °C and the other thermolabile in the freeze-dried state. Repeated cloning and heating of the freeze-dried virus has failed to eliminate the thermolabile population completely but its proportion is substantially reduced. It has also been noticed that rabbit passage increases the thermolabile population. Further studies on the genetics are clearly indicated and are being pursued. Morphologically the thermostable population are white convex non-necrotizing pocks with a penumbra of greyish zone. The thermolabile pocks are flat grey and the size is comparatively smaller on the CAM.

In primary monkey kidney cell culture the plaques formed by the thermolabile element are reticulated and similar plaques formed by the thermostable element are vacuolated. Heating the freeze-dried seed virus at 100 °C alters the flocculating character. The unheated virus produces two distinct precipitating lines, one of which is destroyed by heating.

TESTING OF VACCINE AND SEED VIRUS

Except for the detection of spore bearers, there is no material difference between the test procedure followed in the project and that documented in the WHO Manual no. SA/68.3. Robertson's cooked-meat medium is used for the isolation of spore bearers because (*a*) the ingredients of thioglycollate medium are not readily available and (*b*) Robertson's cooked medium is a standard medium and can be prepared easily. For the innocuity test guinea-pigs are generally used, although in one institute rabbits are employed.

The National Centre cross-checks the initial and stability potency and total bacterial count of a third of the number of batches produced before the finished product is issued to the field. The test results of the production unit are not conveyed to the National Centre. Every 3 months two batches from each institute, tested both at the institute and the National Centre, are sent to the WHO Reference Centre. In addition, sample ampoules are retrieved from the field and tested for potency. The vaccines utilized in the programme are largely manufactured by four indigenous laboratories and in the USSR. There was a precipitous fall in the percentage of batches accepted soon after the International Standard on potency was raised, until finally the quality improved and is sustained. The indices until May 1970 were based on initial potency and bacterial purity. No commitment on stability potency is therefore possible. However, in retrospect, when the comparative results on initial and stability potency carried out since June 1970 are studied, it is valid to presume that earlier batches were predominantly stable as no significant variations were introduced in the manufacturing and test schedules.

The ambient temperature in India during summer is high, while storage and transport facilities are badly developed. The chance of deterioration in the quality of vaccine therefore cannot be ignored. With this in view a study was taken of 41 different batches to compare the infectivity titre at 37 and 45 °C (close to the ambient temperature) after an incubation period of 4 weeks. Appropriate statistical analysis reveals that the regression coefficient is 0·122, which is not significant. The average difference in the log value is

$$\log \bar{y} - \log \bar{x} = 8{\cdot}45 - 8{\cdot}35 = 0{\cdot}10,$$

which is again insignificant. In addition, thermostability studies of the reconstituted vaccine with or without glycerine and at different temperatures and weather conditions were carried out to determine how long the reconstituted vaccine could be used in the field.

The mean potency and stability titre of 60 vaccine batches estimated at the

Table I. *Pock count of the batch tested in groups of 5–6 eggs*

Source of variation	Degree of freedom	Sum of squares	Mean square	*F*	*P*
Between the means of the groups	104	41771·25	401·64	5·75	< 0·1 %
Within groups	439	30366·49	69·85	—	—
Total	543	72137·74	—	—	—

Production Centre, National Reference Centre and WHO Reference Centre have been compared. The initial potency variations were respectively $8{\cdot}72 \pm 0{\cdot}239$, $8{\cdot}65 \pm 0{\cdot}206$ and $8{\cdot}89 \pm 0{\cdot}623$; stability potency variations were $8{\cdot}34 \pm 0{\cdot}187$, $8{\cdot}43 \pm 0{\cdot}217$ and $8{\cdot}31 \pm 1{\cdot}08$ respectively. Similar variations on total bacterial content were respectively $68{\cdot}77 \pm 52{\cdot}03$, $79{\cdot}17 \pm 41{\cdot}26$ and $161{\cdot}0 \pm 135{\cdot}6$. The higher variations in the bacterial count at the WHO Reference Centre were largely due to two batches of vaccine which escaped detection at the Production and National Reference Centre. This unfortunate lapse, however, helped us to locate areas of deficiencies, and since then periodic bacteriological checkup of the nail scrapings, throat and nasal swabs of the technicians directly involved in the manufacture and testing procedures have been enforced.

In quality control, enumeration of pock count on CAM is one of the principal checks. Although wide variation in the technique is an inherent handicap, extremely rigid conditions must be fulfilled before a batch is declared to be standard. With a view to estimating the extent of variations the infectivity titre was performed on one batch 105 times. The data obtained confirm earlier observations. The mean values of the groups of five eggs was between 12·6 and 58·0 and a standard deviation was between 2·23 and 23·69; the coefficient of variation fluctuated between 9·1 % and 76·7 %, though 98 % of the groups had a coefficient of variation less than 50 %. The relevant statistical analysis is summarized in Table I, which indicates that the stability of the pock count is difficult to attain, since not only the individual eggs vary when tested in groups of five, but using the same batch the group means also vary considerably.

COMMENTS

1. The ideal conditions in the manufacture of freeze-dried smallpox vaccine have been discussed. Parenteral use of antibiotics seems to minimize bacterial contamination of the final vaccine to a substantial degree.

2. Parameters other than infectivity titre and limited bacterial contamination need to be formulated to identify the vaccine standard. Such parameters may include data on adequate immunogenicity and negligible reactogenicity of the parent seed and final product. Unlike the manufacture of other vaccines, different seed strains are being used in the manufacture of F.D.S.V. Biological

characteristics of seed strains are reported to undergo changes under different conditions. It is desirable to co-ordinate research programmes to determine whether to retain the present practice of different seed strains or to introduce an international strain throughout the world. India has the unique opportunity of utilizing vaccines manufactured from different seed strains and the field report suggests different grades of reactogenicity.

3. Cross-checking at independent centres ensures maintenance of the quality of freeze-dried smallpox vaccine. The factors relating to storage and communication are major problems in the utilization of vaccine. Random assessment of stability of vaccine batches retrieved from the field and stored in field conditions should form a regular feature in the quality control.

4. Efficiency of an immunizing agent is best estimated by the decline in the morbidity data. Laboratory studies on immunogenicity, therefore, require to be supplemented by field studies.

SUMMARY

Essentially, the manufacturing schedule is similar to that described in the WHO Document SA/68.3. To achieve a highly potent and stable vaccine, potent seed virus, prefreezing below eutectic temperature, early application of maximal heat and maintenance of maximum differential between product and condenser temperature must be ensured.

In quality control initial potency, stability potency and relative freedom from bacterial contamination are principal parameters. Additional criteria on the biological characteristics of seed virus, its high immunogenicity and low reactogenicity are considered extremely important and for this purpose collaborative studies may be formulated.

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A NEW STABLE SMALLPOX VACCINE

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Two types of smallpox vaccines, a freeze-dried and a liquid, are currently in use. Liquid vaccines, on account of their instability, are not suitable for use in countries where smallpox is endemic. However, because liquid vaccines are supplied ready for use, they are frequently preferred by practitioners in non-endemic countries. Freeze-dried vaccines must be rehydrated for use. This is particularly inconvenient with single-dose containers. Although freeze-dried vaccines are stable, they may deteriorate rapidly after reconstitution. Accordingly the World Health Organization recommends that in endemic countries freeze-dried vaccines are not used on the day after reconstitution (3). Even on the same day the vaccine may be inactivated if exposed to sunlight.

In this paper we wish to report on a stable ready-to-use new form of smallpox vaccine. This was achieved by dispersing freeze-dried virus in a water-free semi-solid substance which protects the vaccine from deterioration.

METHODS AND RESULTS

Silicones were chosen as the basis for the vaccine because they are inert, water-free and water-repellent. Extensive studies in animals and man have shown silicones to be non-toxic. During the past 20 years silicones have been used in foods, drugs and cosmetics (2, 4). For our studies we used Dimethylpolysiloxanes DC 200 (Dow Corning Corporation, Midland, Michigan), and our own freeze-dried smallpox vaccine 'Lancy-Vaxina', which contains the Lister strain of vaccinia virus (1). This vaccine was ground to a fine powder in a ball-mill.

With a special method, for which a patent was granted, the freeze-dried virus was dispersed into the silicone of high viscosity (60 000 centistokes). One gram of the ointment-like vaccine thus obtained contains an equal number of virus particles as 1 ml of the reconstituted freeze-dried virus suspension. The ointment vaccine was stored at approximately +4 °C until used. The silicone smallpox vaccine was subjected to the following tests:

STERILITY AND INNOCUITY TESTS

They were performed with the lyophilized vaccine used for the preparation of the ointment vaccine as well as with the ointment vaccine itself. They were satisfactory.

Table I. *Rabbit skin titration of the silicone vaccine*

Vaccine	Unheated		Heated (4 weeks at 37 °C)	
	1:1000*	1:10000	1:1000	1:10000
No. 1	C†	4‡	C	2
No. 2	C	6	C	8

* Vaccine dilutions.
† Confluent lesions.
‡ Number of vesicles on rabbit skin.

POTENCY AND STABILITY TESTS

They were performed with the end-product only. Since the silicone-ointment vaccine cannot be dissolved in water or aqueous solutions in order to prepare the further dilutions required for standard potency laboratory tests, its potency was determined with the rabbit skin test(**5**).

The vaccine was diluted with low viscosity (0·65 centistokes) silicone fluid; 0·1 ml aliquots of a 1000- and 10000-fold diluted vaccine were applied to 2·5 × 5·0 cm areas of scarified skin. Six days later the vesicles were counted. The results of two experimental vaccine batches are presented in Table I. Based on former WHO requirements for the rabbit skin test, both vaccines were potent and stable(**6**). The vaccine was stable for 12 months at approximately 4 °C. Long-term stability tests are not yet completed.

Vaccine dilutions in silicone oil cannot be used for titrations in eggs because the oil damages the chorioallantoic membranes. Methods for an optimal re-extraction of the incorporated viruses are currently investigated.

CLINICAL TRIALS

Clinical trials with the silicone vaccine were performed at the Institute for Social and Preventive Medicine at the University School of Medicine in Zurich. The vaccinations were performed using the scratch technique. The take rate and the local reactions were comparable to those found with the lyophilized vaccine(**5**). From both the rabbit-skin test and the clinical trial we can conclude that the silicone does not interfere with the uptake of the virus by the host cells. This might be explained by penetration of the silicone fluid through the skin(**1**), the virus being left on the surface.

SUMMARY

A new ointment-like smallpox vaccine was developed by dispersing freeze-dried virus in silicone. The vaccine can be applied by any vaccination technique based on skin scarification. The silicone-incorporated freeze-dried viruses are protected from deterioration. The potency and stability of the ointment vaccine has been shown by laboratory tests and clinical trials. The obvious advantages of the new

ointment-type vaccine are that it is ready for use and it requires no rehydration as does the lyophilized vaccine. It can be filled, stored and transported without sealed containers and it can be easily distributed in single-dose containers.

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Discussion

ESPMARK (Sweden) The label on the bottles of silicones that we have used for other kinds of experiments show warnings not to drop it on the skin because it can cause blisters and even worse reactions. What concentration did you use in your experiments when adding silicon to smallpox vaccine? One might expect blisters and other reactions that are due to silicon itself.

MAJER (Switzerland) The silicon that we have used is inert, showing no reactions on the skin of laboratory animals.

NETTER (France) (1) As a Frenchman, I am pleased to see that Doctor Majer is using for titration Calmette's method of rabbit scarification, but how is it in agreement with WHO recommendations to check on CAM? (2) What about the addition of Tween monoleate for the dilution of vaccine for titration?

MAJER (Switzerland) (1) Methods for titration on CAM are currently studied. (2) Not done, but I think it will not work with silicon.

MARENNIKOVA (USSR) (1) Have you any experience in virus titration in different ampoules of the same series of vaccine? (2) Are there any developments in preparation of the silicon vaccine for potency test?

MAJER (Switzerland) (1) Our experience is so far limited. This will be studied next. (2) Yes, a CAM titration method will be worked out in the near future.

BENENSON (USA) Would you please explain the method of application of the ointment to the skin? Could it be used by the multiple puncture method?

MAJER (Switzerland) The ointment vaccine can be applied by scarification or multiple puncture method.

TINT (USA) (1) Will the silicone vaccine be affected by variations in the residual moisture of the freeze-dried material that is incorporated in the vehicle; is there an upper moisture limit? (2) If the vaccine is applied to the bifurcated needle, are the adherence characteristics well enough known to assure uniform dosage?

MAJER (Switzerland) (1) The properties of the freeze-dried virus including the residual moisture will determine the stability of the ointment vaccine. (2) To assure uniform dosage on the bifurcated needle as a single-dose container, modifications of the needle might be necessary.

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THE FREEZE-DRYING OF SMALLPOX VACCINE ON SINGLE DOSE APPLICATORS AND THEIR USE WITHOUT RECONSTITUTION

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Despite all efforts, the technique of smallpox vaccination has not basically changed since the early days of its introduction. Modified live or inactivated vaccines are not yet in general use and modern methods of application have only recently been discussed. So, at least for the near future, we shall have to be content with the conventional epicutaneous application of vaccine.

Due to the tremendous efforts of the WHO eradication programme, the vaccination policy in central Europe has been subject to changes. Mass vaccinations, formerly the predominant form of smallpox immunization, are gradually being replaced by individual vaccinations. A recent inquiry conducted at our Institute revealed that only 40% of all primary vaccinations are performed by the health authorities, whereas 60% are done by private practitioners or paediatricians, most of these vaccinations being carried out individually. Consequently, the multidose container, once the predominant form of vaccine distribution, is in decreasing demand.

A second line of development is the gradual substitution of liquid vaccine by a freeze-dried product with its undoubtedly better stability. It may be stated that no liquid vaccine being shipped over any distance ever reaches its destination with a potency equal to that of a lyophilized product. Drying and subsequent reconstitution, however, require a certain minimal volume to be manageable. This volume usually exceeds by far the amount of vaccine actually needed. Thus, more vaccine must be produced than is required, which limits the routine employment of purification procedures with their high loss of virus.

A third assumption is based on the evident reluctance with which a dried product is accepted, because it requires, in contrast to liquid vaccine, cumbersome reconstitution.

Finally, smallpox vaccination requires the use of a special instrument which is certainly not always properly sterilized, thus permitting transmission of infectious agents, such as the one related to hepatitis.

It follows that, for the use of the private physician, the vaccine should meet four requirements. (1) It should be available in single-dose containers. (2) It

should be freeze-dried. (3) It should not require reconstitution. (4) It should be dispensed by a disposable applicator.

Innumerable applicators for smallpox vaccine have been suggested in the history of vaccination. Apart from the usual needles and lancets, special mention should be made of the Kravitz ring, the bifurcated needle and the perpunctor – three devices that have been suggested and successfully used in recent years.

Our own applicator consists of a round plastic rod, carrying at one end eight spikes arranged in a circular position and thus forming a central cylindrical boring. This arrangement allows one to pick up a drop of fluid which will fully cover the tips of the spikes without much adherence to their outer surface. Thus, very little material is deposited outside the actual site of vaccination. An additional function of the spikes is to hold mechanically the plug which forms after freeze-drying. Thirdly, of course, the spikes must cause enough damage to the skin to ensure infection and thus a sufficient take-rate.

Different types of applicators were compared for optimal results. The principal idea was to use a cone-shaped rod with a concentric boring and a sharp circular edge, acting like a punch. Upon firm application, the skin bulges into the central boring, being stretched and superficially damaged around the circular edge. Difficulties arise in holding the plug of dried vaccine in place, since it tends to fall out after the slightest shrinkage. The edge was therefore broken up by radial incisions. The effect of different types and arrangement of spikes, among them the Kravitz ring, were tested on animal skin. Non-immune and immune rabbits, sheep and calves were used. In principle, different dilutions of the same vaccine were deposited in single drops. Each applicator was tested on each dilution of vaccine by pressing it firmly through the deposited volume into the skin. The effectiveness of the applicator was judged from the final dilution of vaccine giving a major reaction.

All applicators gave essentially similar results. We therefore chose the one which incurred minimal damage and thus caused minimal pain. The eight spikes are formed by shaping the plastic rod (diam. = 3 mm) to a cone at an angle of 25° and giving it a concentric boring with eight axial incisions. The spikes are 2 mm long and stand 0·6 mm apart. They hold approximately 0·01 ml of vaccine. The rods are washed, dried and sterilized by ethylene oxide. They are then mounted into the central hole of a freeze-drying stopper and charged by dipping them into the liquid vaccine. No additional stabilizer is added to the 5 % peptone in McIlvaine buffer. After insertion of the stoppers into the neck of a regular vial, the freeze-drying process is performed as usual. The product is frozen at −45 °C for 2 h and then heated to +20 °C. The drying is completed after 24 h with a final vacuum of less than 10^{-3} torr. Titres above 10^{8} pfu/ml can be obtained, assuming an average volume of 0·01 ml per applicator.

The charged applicator may be used without reconstitution. Firm pressure causes enough exudation to reconstitute a sufficient quantity of vaccine.

Our results so far comprise 419 revaccinations in human beings. Most of them were school children aged 12 years. The results, as compared to a control group vaccinated with the conventional scratch technique, are shown in Table I. The local lesions seemed to be more confined than after scratch-vaccination. Result-

Table I. *Revaccination results with a disposable applicator compared to conventional scratch technique*

Method used	Total number vaccinated	Result	
		Negative	Positive
Applicator	419 (100 %)	52 (12·4 %)	367 (87·6 %)
Scratch technique	2042 (100 %)	23 (1·1 %)	2019 (98·9 %)

ing scars tended to be small. The higher incidence of negative results may be eliminated by a higher potency of the vaccine. The economy of production and the ease of performance seem to justify further development.

SUMMARY

Smallpox vaccine is freeze-dried in volumes of 0·01 ml on to the spikes of a disposable plastic applicator. The charged applicator permits individual vaccinations without reconstitution of the freeze-dried product. The applicator is firmly pressed to the skin at the site of vaccination. Small amounts of exudate suffice to reconstitute enough material for successful vaccination.

Discussion

NETTER (France) (1) How many individual doses can be processed in one freeze-drying operation? (2) There is economy of pulp but not of money because it will be less expensive to use 50 doses processed by the classic method.

HOCHSTEIN-MINTZEL (West Germany) (1) 4000.

STICKL (West Germany) Did you compare different methods of vaccination in respect of painless vaccination?

HOCHSTEIN-MINTZEL (West Germany) We have shown that this method, in comparison with other methods, is painless. It seems therefore that it is the method of choice for vaccination of children.

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EFFECT OF LIVE AND INACTIVATED VACCINIA VIRUS PREPARATIONS ON PRODUCTION OF ANTIBODIES IN ANIMALS

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This paper describes antibody studies where guinea-pigs were injected with β-propiolactone and ethyl alcohol inactivated as well as live vaccinia virus preparation. The sera were analysed by haemagglutination-inhibition tests. Similar results were obtained with rabbits and monkeys. Comparative studies of purified live vaccinia virus preparations emanating from sheep skin and chicken-embryo membranes were performed. The production of antibodies in guinea-pigs was studied with and without the addition of adjuvant to virus preparations.

MATERIALS AND METHODS

Virus preparation The strain of vaccinia virus used was the Lister-strain passed twice through embryonated eggs.

The crude virus preparation was purified by homogenization, treatment with deoxyribonuclease I, gel chromatography and, finally, concentrated by ultracentrifugation.

Inactivation of the purified vaccinia virus preparations by β-propiolactone and ethyl alcohol The purified vaccinia virus preparations used were inactivated with β-propiolactone and ethyl alcohol according to the method described previously(**6**).

Titration of pock forming units The preparations were tested for infectivity by inoculating 0·1 ml amounts of serial tenfold dilutions on the cavitational surface of the chorioallantoic membranes of 12-day-old chicken embryos.

The titres were expressed as pock forming units per millimetre (pfu/ml).

Haemagglutination-inhibition test The method described by McCarthy & Helbert(**13**) was used.

Studies of the effect on rabbits caused by uninactivated and inactivated purified preparations Purified vaccinia virus preparations (titre $10^{8\cdot0}$ pfu/ml) and purified and ethyl alcohol and β-propiolactone inactivated vaccinia virus preparations (titre before inactivation $10^{8\cdot0}$ pfu/ml) were injected intravenously in one ear (1 ml/animal) and ten animals were used per experiment. The animals were followed daily during a period of 3 weeks.

Measurement of haemagglutination-inhibiting antibodies Purified live vaccinia virus preparations were diluted from the titre of $10^{8\cdot0}$ pfu/ml down to 10^{1} pfu/ml and injected into the animals. The animals were injected with 1 ml/animal and for each dilution step 20 guinea-pigs. Three weeks after injection the haemagglutination-inhibition antibody titres were determined.

Antibody studies on guinea-pig sera The antibody titres of 250 guinea-pigs (50 in each group) were studied by haemagglutination-inhibition test during a period of 2 years. The group 'PP' (purified vaccinia virus preparation) was injected intramuscularly (1 ml/animal) with a live vaccinia virus preparation (titre $10^{8\cdot0}$ pfu/ml). The groups 'PP-ALC' (purified vaccinia virus preparation inactivated with ethyl alcohol) and 'PP-BPL' (purified vaccinia virus preparation inactivated with β-propiolactone) were injected at the same time with purified inactivated vaccinia virus preparation (1 ml/animal). After 3 weeks the booster was given to the three mentioned groups. The booster on 'PP' was live virus preparation and with the 'PP-ALC' and 'PP-BPL' groups inactivated virus preparations were used. The volume of preparations injected as booster was the same per animal as mentioned above. Three months after the start of the three groups and half of the control group mentioned normal control, 'NC' (earlier injected with physiological saline), were scarified on an area (5 × 5 cm) of the skin of the guinea-pig's flank and then inoculated with 1 ml of a living vaccinia virus preparation (titre $10^{8\cdot0}$ pfu/ml).

Seven days later the pocks were counted. The other half of the control group mentioned normal control's control 'NCC', was scarified and inoculated with physiological saline. One week after scarification the pocks were counted.

Effect of adjuvant on antibody titre of guinea-pig sera The antibody titre of guinea-pigs was studied by haemagglutination-inhibition test. Guinea-pigs were injected with purified preparations which were inactivated with ethyl alcohol and β-propiolactone. To the inactivated preparations were added sodium phosphate (Na_2PO_4) and aluminium-chloride ($AlCl_3$) as adjuvant. The final pH was 7·2.

Purified inactivated preparations without adjuvant were also used, as well as a normal control (animal injected with physiological saline). Guinea-pigs were injected with 1 ml per animal and the haemagglutination-inhibition antibody titres were studied after 1, 6, 12 and 18 months. One hundred animals were used – that is, 20 in each group.

Comparison of the HI-antibody production caused by a purified vaccinia virus preparation grown on egg membrane or on sheep skin Studies of the character of the HI-antibody production elicited by purified vaccinia virus (Lister strain) grown on egg membranes compared with purified vaccinia virus (Lister strain) grown on sheep skin have been done. Twenty monkeys were injected in each group (1 ml/animal). As a normal control ten monkeys were injected with physiological saline (1 ml/animal). The antibody titres were followed by haemagglutination-inhibition tests for 3 years.

RESULTS

Studies of the effect in rabbits caused by uninactivated and inactivated purified preparations At the beginning of a 3-week period rabbits were injected intravenously in the ear (1 ml/animal) with a purified-uninactivated as well as ethyl-alcohol and β-propiolactone-inactivated vaccinia virus preparation. During this period the animals showed no signs of pathological changes.

Measurement of haemagglutination-inhibiting antibodies The guinea-pigs showed no haemagglutination-inhibition antibody titre after 3 weeks when injected with vaccinia virus preparations with a titre under 10^4 pfu/ml.

Antibody studies on guinea-pig sera As can be seen in Fig. 1 (*A–C*), the scarification and the inoculation on the skin of the guinea-pigs in the different groups took place on the 91st day, after the first injection and the counting of the pocks

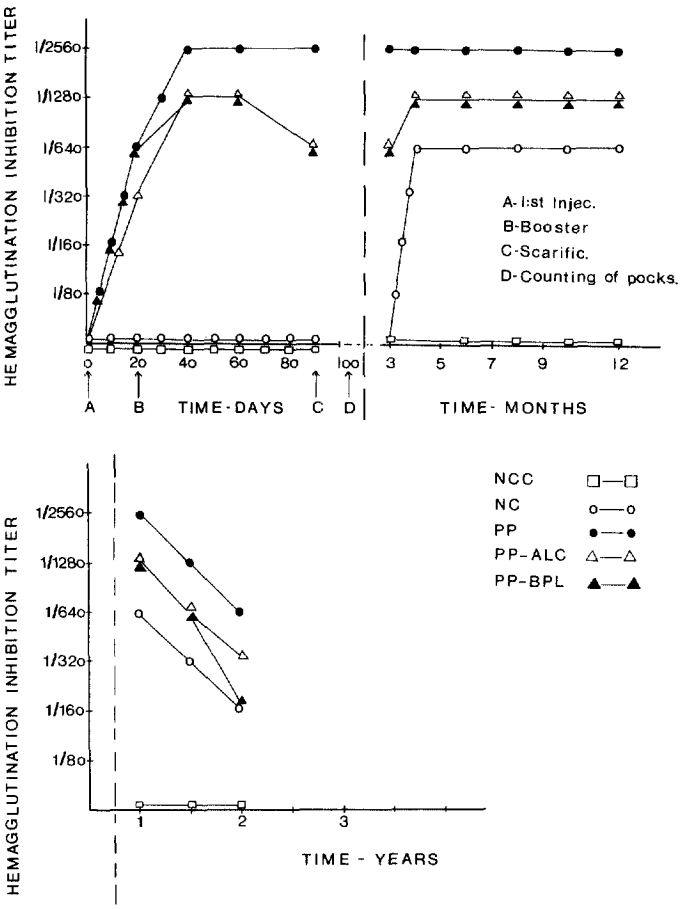

Fig. 1. Antibody studies on guinea-pig sera during a period of 2 years.

on the 98th day. The mean values of the pocks of the groups were: PP 3 pocks, PP-ALC 5 pocks, PP-BPL 10 pocks, NC 24 pocks and NCC 0 pocks.

In each group there were 50 guinea-pigs except the NC, 48, and NCC, 45 guinea-pigs, where 2 and 5 respectively had died during the experiment. The results of the haemagglutination-inhibition are also shown on Fig. 1 (*a–c*). The PP gave after booster the titre 1/2560 while the PP-ALC and PP-BPL groups stopped at 1/1280. Before scarification the PP-ALC and PP-BPL were going down to 1/640, but after scarification both of them went up again to 1/1280. After 1½–2 years all the groups are going down in antibody titre.

Effect of adjuvant on the antibody titre of guinea-pig sera Fig. 2 shows the haemagglutination-inhibition titre 1/640 after 1 month on the PP-ALC (purified preparation inactivated with alcohol) and the PP-ALC-AD (the same type of preparation with adjuvant). After 6 months the titres were unchanged on the

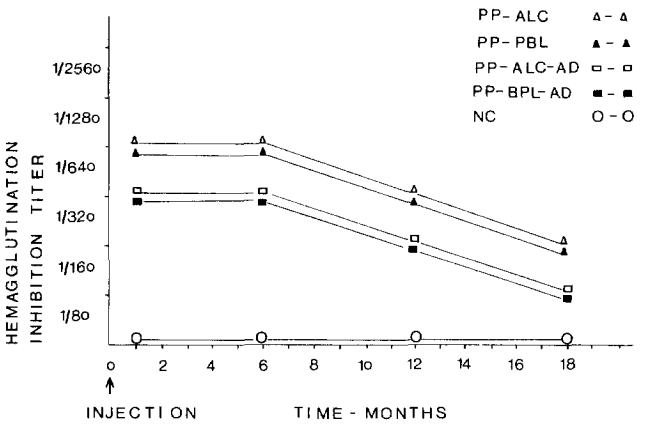

Fig. 2. Effect of adjuvant on antibody titre of guinea-pig sera during a period of 18 months.

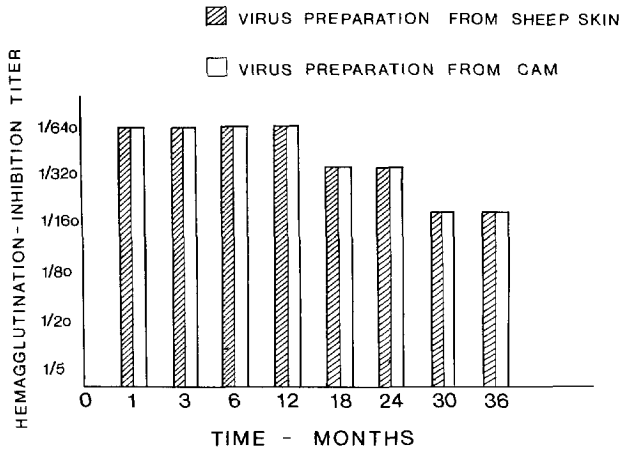

Fig. 3. Comparison of the HI antibody production caused by a purified vaccinia virus preparation grown on egg membrane or on sheep skin.

two groups while after 12 months the titres were 1/320 and after 18 months 1/160.

The preparations PP-BPL (purified preparations inactivated with β-propiolactone) and the PP-BPL-AD (the same type of preparation with adjuvant) had the titres 1/320 after 1 month. After 6 months the titres were still 1/320, but after 12 months 1/160 and after 18 months 1/80. As can be seen in Fig. 2, there were no differences in the titres between the groups with and without adjuvant, but a small difference between ethyl alcohol and β-propiolactone inactivated preparations.

The normal control gave no titre.

Comparison of the HI-antibody production caused by purified vaccinia virus preparations grown on egg membranes and on sheep skin In Fig. 3 mean values of the haemagglutination-inhibition titres of preparations from egg membranes and sheep skin are the same. After 1 month they are 1/640 and remained there to the 12th month. After $1\frac{1}{2}$ years they went down to 1/320 and after $2\frac{1}{2}$ years to 3 years down to 1/160.

Studies in other animal species Similar results as described for guinea-pigs were obtained with rabbits and monkeys.

DISCUSSION

Madely(**11**) pointed out that most authors describe the quantity of neutralizing antibody produced under different experimental conditions but few talk about the nature of produced antibody.

Collier *et al.*(**4**) showed that when vaccinia virus is inactivated by ultraviolet irradiation the margin between inactivation of the virus and the loss of antigenicity is narrow. According to the writer the method of inactivating vaccinia virus using β-propiolactone or ethyl alcohol have not shown such problems.

Interesting observations were made by Kaplan *et al.*(**10**), who found typical 'takes' after vaccination with live vaccine on persons earlier vaccinated three times with inactivated vaccine. The writers are doubtful if the inactivated vaccine may protect against variola. This is, of course, a difficult question, but the aim of this paper has been to obtain a basic immunity using an inactivated virus preparation and then use living virus for increasing the immunity. Kaplan has earlier published(**9**) a paper about an ultraviolet-irradiated vaccinia virus vaccine. The vaccine was injected intramuscularly on rabbits and guinea-pigs and gave no haemagglutination-inhibiting antibody but neutralizing antibody. In spite of high titre of neutralizing antibodies in sera after vaccination with inactivated vaccine, a strong reaction was obtained when vaccination with living virus was done.

Contrary to these results haemagglutination-inhibition antibodies were obtained in the present study, when β-propiolactone and ethyl alcohol were used as inactivators of virus. The reactions were smaller when live virus followed an injection with inactivated preparation.

Mahnel(**12**) admitted that the relationship between serum antibody and tissue immunity was not quite distinct. In spite of low serum antibody titre the tissue immunity existed and vice versa. Neither is the ratio between neutralizing titre and immunity confirmed. Positive haemagglutination-inhibition antibody titre probably gives immunity. The absence of discovery of haemagglutination-inhibition antibody does not eliminate the presence of immunity in rabbit.

Boulter(**1**) declared that the immunity obtained by injection with an inactivated vaccinia virus preparation was probably of a non-humoral nature and the high-titred neutralizing antibody is an irrelevant side-effect of the vaccine.

Boulter pointed out that the cellular immunity has at least the same importance as the humoral immunity in protection against poxviruses.

It is questionable if the obtained high neutralizing-antibody titres have any bearing on good immunity. However, further experiments are needed to relate both titres of neutralization and haemagglutination-inhibition to each other as well as to immunity against infection.

SUMMARY

The antibody titres on guinea-pigs, rabbits and monkeys were studied by haemagglutination-inhibition tests, after injection with β-propiolactone- and ethyl-alcohol-inactivated as well as living purified vaccinia virus preparations, during a period of 2 years.

A vaccinia virus preparation from sheep skin has been compared with one from CAM on the ability of building haemagglutination-inhibition antibodies during a period of 3 years. The effect of adjuvant on guinea-pigs has also been studied.

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Discussion

HOCHSTEIN-MINTZEL (West Germany) What parameters for the immunogenicity of the inactivated vaccine are there except HI antibodies in the species mentioned?

HEDSTRÖM (Sweden) Other parameters have not been investigated as yet.

RICHTER (West Germany) (1) Did you have 86 % recovery of the virus in your vaccine? (2) If you do no protein estimation how can you say that your virus is purified?

HEDSTRÖM (Sweden) (1) Yes, on the purification process we have up to 86 % yield. (2) We have done protein estimation but I could not show all the details.

KAPLAN (UK) How does Hedström's vaccine fit in with the findings of Appleyard that the protective antigen of inactivated vaccinia virus resides in the extracellular fraction of virus, and that the intracellular virus does not significantly provide any useful antigen? Hedström's virus, extracted from chorioallantoic membranes, must have consisted of up to 77 % of intracellular virus.

HEDSTRÖM (Sweden) I only refer to my obtained results. Further studies can be done to find out the reason for the divergent results obtained.

EHRENGUT (West Germany) My question is in line with Dr Kaplan's comment. In human beings, immunized with formalinized non-infectious smallpox vaccine from extracellular and intracellular origin, we never found HI antibodies. Has it been proved that in the study of Dr Hedström the virus was absolutely inactivated? In another study in guinea-pigs immunized with formalinized non-infectious vaccine (from the Behring Company) a serological response was seen very rarely.

HEDSTRÖM (Sweden) There can be several factors influencing the obtained results – for example, the character of the strain and the way of purification and inactivation. β-Propriolactone and ethyl alcohol has been used instead of formaldehyde and may be one of the factors. The inactivation procedures are giving an absolutely inactivated virus which is confirmed by different control tests.

EDSALL (UK) What do we have to date on which to base the assumption that serologic – rather than cell-mediated – immunity can be used as a criterion of the efficacy of a smallpox vaccine, either live or inactivated.

HOCHSTEIN-MINTZEL (West Germany) Today it is an open question and it is time to solve this problem and find the acceptable method or methods.

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PRODUCTION AND USE OF SMALLPOX EGG VACCINE

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Since 1958 the Biological Research Institute (BRI) of the Secretariat of Health of the State of Rio Grande do Sul, Brazil, has been producing smallpox vaccine prepared in the chorioallantoic membrane of chicken embryos (**3, 4**).

As far as we know, ours is the only laboratory in the world which produces this kind of vaccine on an industrial scale. In 1970 we produced 26920000 doses for the Smallpox Eradication Campaign (SEC) and in 1971, 34060000 doses.

The production of smallpox egg vaccine offers large advantages, such as minimizing the operational installations, low cost, sterility, and the possibility of rapidly increasing the output in emergencies. Aspects that formerly had been subject to doubt, such as immunological and infectious response, innocuity, and heat-stability have been studied by many authors (**1, 2, 5, 6**), who proved that the results were comparable for tests made with vaccines produced in calf lymph and in chicken embryos.

TECHNIQUE OF PRODUCTION

Fertile eggs are obtained from a chicken farm where the reproducing stock is under permanent veterinary control. The eggs are incubated at 36 °C, 80 % moisture, with automatic turning every 2 h. On the 12th day the eggs are candled in a darkened room and during this operation the polar cap and a lateral spot of the shell are perforated without antisepsis. Suction from a small vacuum pump on the polar hole causes the appearance of an artificial air-sac with exposure of the CAM. The third embryo passage seed virus for inoculation comes from the Lister Institute, Elstree, England. The titre of the seed solution is 40·00 i.u. per 0·2 ml. Two hours after candling each egg is inoculated with 0·2 ml and the perforation is sealed with adhesive tape. The diluent is powdered milk at 5 % (2 ml), McIlvaine buffer 0·32 M (2·5 ml), and sufficient water to complete 200 ml.

The eggs are incubated at 36 °C during 48 h in a stationary position. After renewed candling the live egg embryos are exposed to ultraviolet radiation for 10 min. The harvest of the CAM is performed by placing one unit per test-tube and changing the set of sterilized scissors and forceps after each series of 18 eggs. While using one set of scissors and forceps the other is immersed in ethanol at 70 % and sterilized distilled water. The individual CAM's are tested for sterility in thioglycolate and are then kept in a freezer at −26 °C. The sterile membranes are placed together, weighed, homogenized in a Sorvall Omnimixer, and titred. For the preparation of one batch the final theoretic titre is calculated at $10^{8.70}$. Half of the total volume is obtained by adding PBS, and this volume is doubled by adding peptone at 10 %. The distribution into vials is effected automatically, being followed by placing slotted butylic rubber stoppers on the containers. The vials,

placed on perforated trays, are immersed in ethylic alcohol surrounded by dry ice for 10 min and transferred to Repp Sublimators. At the end of the drying operation the internal and external pressures are balanced by means of injecting oxygen-and-moisture-free nitrogen, and the stoppers are driven home by means of pressure plates while still in the drying chambers. After this a semi-automatic machine seals the vials with aluminium caps. Before and after freeze-drying each batch is subjected to tests of sterility and potency (CAM titre). Further tests are made for heat-stability (titration of the vaccine after holding it at 37 °C for 28 days) as well as a test vaccination in primary and re-vaccinated humans. Four times a year we send samples from batches to the Connaught Medical Research Laboratories, Canada, where, in addition to the tests carried out by us, research is done on innocuity with guinea-pigs and rabbits, plus potency tests by scarification on rabbits, with further determination of the residual moisture.

CONSIDERATIONS

Since the start of the production of smallpox egg vaccine several changes have been tried. Several of these modifications have the aim of placing our vaccine within the requirements of potency, stability, and purity required by the World Health Organization.

After several tests we selected bacteriological neutralized peptone as conservant, and the one supplied by Oxoid was chosen because of its neutral reaction, absence of phosphates, and quick solubility.

The modifications made in the manner of candling, with a view of having the egg ready for inoculation with one only manipulation, permitted us to increase production while effecting a simultaneous reduction of man-hours, and a further improvement of the rentability by increasing the number of pocks, while the average weight of the membranes changed from 1 to 1·8 g per egg.

The devices adapted to the tables of the cabinets, which hold the egg while being opened and carry the discarded material away from the operators, allow manipulating 1200 eggs weekly by only four operators, and with contamination indices below 2%.

A pre-freezing operation was added to the freeze-drying cycle. The bottoms of the sublimator trays were perforated in order to obtain quicker and more homogeneous freezing in the bath of alcohol and dry ice. We changed the cycle of freeze-drying, adding a period of 4 h to the refrigeration of the product and a period of 10 h after it has reached ambient temperature, totalling an average of 36 h. As a result of these changes we have reached contents of residual moisture below 0·5%, frequently attaining 0·1–0·3%.

After freeze-drying, the titres of the vaccines are above $1 \times 10^{8\cdot10}$ and the decreases of these titres in the heat-stability test are 0·3–0·5 log. In a total of 11 months in 1971 we were able to produce 34060000 doses with a staff that worked half-time and never exceeded nine in number. By using vials with a smaller diameter – which have already been ordered – we shall be able to increase our annual production in case of need.

The vaccine is supplied in vials of 50, 100 and 200 doses and reconstitution is made by adding water, distilled or glycerinated at 30%; administration,

according to the norms of the WHO may be made with bifurcated needles or with jet injectors.

The efficacy of this vaccine, correctly administered on a large scale, was evaluated during a field test carried out by the team of the Epidemiological Control Unit of the Secretariat of Health of Rio Grande do Sul (ECU), in the county of Soledade, in April 1970; 230 vaccinations being made, of which 65 were first vaccinations. The percentage of takes was 100% both for the vaccinated and the revaccinated persons.

During the Smallpox Eradication Campaign (SEC), often under unfavourable conditions of preservation and administration of the vaccine, data were collected in 51 counties of the State of Rio Grande do Sul from April to December 1970;

Age-group	Readings	Takes	Percentages of takes
3 months to 4 years	8016	7836	97·75%

INNOCUITY

Some authorities manifest fear of the transmission of fowl viruses by means of the percutaneous inoculation of smallpox egg vaccine. Brazil has been producing an injectable yellow-fever vaccine in chicken embryos for many years, and no relationship between oncogenesis and vaccination could be found in the use of both vaccines. All batches of our smallpox vaccine are sterile from a bacteriological point of view, and always prove to be innocuous in tests on guinea-pigs and rabbits.

The incidence of post-vaccinal complications with smallpox egg vaccine may be evaluated by means of the data of the Smallpox Eradication Campaign and of the Epidemiological Control Unit of the Secretariat of Health: vaccinations effected by the SEC in Rio Grande do Sul during 1970–1 number 6527372; information on post-vaccinal complications received by the ECU during that period are as follows:

	cases
Encephalitis	2
Eczema vaccinatum	2
Vaccinia	1
Total	5 (all with favourable outcome)

The immunogenic capacity of the vaccine produced by the BRI has made it possible to change the situation of the State of Rio Grande do Sul, which was formerly considered an endemic smallpox area. The data that follow are supplied by the SEC:

Number of smallpox cases in Rio Grande do Sul

1970 (during vaccination by SEC)	932
1971/72 (up to September 30)	0

At present this vaccine is being used by the Ministry of Health in 90% of the

vaccinations in our country, having given a decisive contribution to the eradication of smallpox in Brazil:

Number of smallpox cases in Brazil

1969	6676
1970	1771
1971	19
1972 (up to Sept. 30)	0

The vaccine is highly economical. For the production of 3 million doses per month we use a team of only nine persons. The cost of the vaccine, not including the invested capital, totals about US $2.00 per 1000 doses. The smallpox vaccine produced by the BRI has been delivered to all Brazilian states, and at the request of the WHO shipments were made to Surinam, Haïti, Mexico, Puerto Rico, Honduras, Yugoslavia, and Germany.

The equipment of BRI's smallpox-vaccine-producing laboratory is being supplied by the WHO.

A large numer of the modifications the author has introduced in this laboratory were the result of a fellowship given by the WHO which permitted him to make studies of smallpox vaccine production at the Connaught Medical Research Laboratories, Canada, and to visit the similar laboratories of the Instituto Nacional para Programas Especiales de Salud, Colombia, and the Instituto Carlos Malbran, Argentina.

SUMMARY

The Biological Research Institute of Rio Grande do Sul has been producing smallpox egg vaccine for many years.

According to our information, it is the only laboratory in the world producing this kind of vaccine on an industrial scale.

Many modifications of the process were introduced in order to make it practical and economical, as well as to obtain the production of a vaccine with high potencies after freeze-drying and heat-stability tests.

The vaccine, which by now is being used in 90% of all vaccinations in Brazil, has been evaluated with regard to immunity, innocuity, and efficacy during the Smallpox Eradication Campaign that has exterminated smallpox in Brazil since May 1971.

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Discussion

LEMOINE (Belgium) (1) Are the eggs 'specific pathogen free' (SPF)? (2) What is the titre of the raw material (MCA as harvested)? (3) Is any antiseptic added to the vaccine?

VOEGELI (Brasil) (1) No. (2) The titre of the pulp is about $1 \times 10^{10 \cdot 0}$. (3) No antiseptic is added to the vaccine.

PERKINS (UK) What do you do about fowl leucosis viruses?

VOEGELI (Brasil) We use fertile eggs obtained from a chicken farm where the reproducing stock is under permanent clinical veterinary control.

EVANS (UK) I would like to ask Dr Henderson the question: Is the smallpox vaccine prepared in Brazil accepted and recommended by WHO in its eradication campaign?

HENDERSON (WHO) Each country must, of course, decide its own licensing requirements. Vaccine donated to WHO must meet the requirements laid down by the WHO Expert Committee on Biological Requirements to be accepted for use. Dr Voegeli is troubled, as are we, by the relative instability of his vaccine – a number of lots, as he points out, containing less than 10^8 pfu/ml after incubation for 4 weeks at 37 °C. If the vaccine in Brazil is properly handled, as clearly it has been, both primary and revaccination take-rates may be perfectly satisfactory with a vaccine of this titre, but clearly it must be handled more carefully than a vaccine which fully meets the WHO requirements.

Session II

GENERAL DISCUSSION

Reply to the question on acceptability of egg vaccines for the WHO programme

As pointed out by Dr Voegeli, yellow-fever vaccine has been produced and employed in Brazil and other countries for many years. In vaccination a substatial quantity of vaccine is administered subcutaneously. Neither in Brazil nor in other countries is it required that this vaccine be produced in eggs obtained from flocks free of recognized adventitious agents. Considering that this is the situation in regard to yellow-fever vaccine, should the government of Brazil require higher standards for smallpox vaccine given intradermally in a smaller quantity or should WHO, in continuing need of vaccine, refuse to accept smallpox vaccine produced in eggs?

ESPMARK (Sweden) In reply to Dr Henderson I would like to point out that the titres of Dr Voegeli's vaccine were of the order of 7·7–7·8 and even higher; and as we have heard, both in Brazil and by reports, the take-rate in primary vaccinees is quite satisfactory. On the other hand, I agree that when the surveillance phase comes and revaccination is common there will certainly be a need for a stronger vaccine, e.g. of 10^8 inf. units/ml.

HENDERSON (WHO) The WHO recommended standards were established by the Expert Committee on Biologics Standards following a series of collaborative studies conducted during the early 1960s. It was recognized that a high proportion of successful takes could be obtained among unvaccinated persons with vaccines which had a potency of only $10^{6·0}$ to $10^{6·5}$ pfu/ml. However, for newborns and those receiving revaccination, a vaccine with a titre at least 1 log higher was required. Thus it was decided that vaccines should contain $10^{8·0}$ pfu/ml. As the vaccine is often subjected to ambient temperatures under field conditions, it was further decided that the vaccine should contain this quantity of virus after incubation for 4 weeks at 37 °C.

All eradication programmes, from their inception, involve vaccination of the unvaccinated, revaccination especially of contacts of cases, and vaccination of newborns. Thus it is absolutely necessary, from the inception of programmes, to have vaccine meeting the requisite standards. As earlier noted, the principal costs in a programme are involved in reaching the recipient. We must be certain that when we reach a population for vaccination, the vaccine is fully potent. A compromise in standards is simply not possible.

MARENNIKOVA (USSR) Have you any observation on postvaccinal complications (encephalitis) after application of the egg vaccine?

VOEGELI (Brazil) We had only two cases of postvaccinal encephalitis per 6·5 million vaccinations, and both with favourable outcome.

BENENSON (USA) What strain of virus was used to prepare your vaccine?

VOEGELI (Brazil) The strain of virus used by us comes from the Lister Institute and is in the third embryo passage.

STICKL (West Germany) You reported on only two cases of postvaccinal encephalitis after more than 6 million vaccinations. That is a quite lower rate than observed in other countries. Can you give any suggestion why you got such a good result?

VOEGELI (Brazil) These data were furnished by the Epidemiological Control Unit of the Secretariat of Health after wide use of egg vaccine in the State of Rio Grande do Sul.

RICHTER (West Germany) I have several questions about allergic complex. (1) Have you any control system to observe complications? (2) Have you observed persons who have had allergic reactions (local or common) after first vaccination and after revaccination? (3) Have you tested by vaccination persons who have egg-allergy? (4) Do you use the jet-injection?

VOEGELI (Brazil) (1, 2) The Secretariat of Health of the State of Rio Grande do Sul has an Epidemiological Control Unit that worked during the Smallpox Eradication Campaign making readings, observations and collecting data. (3) No. (4) Jet-injectors were widely used during the Smallpox Eradication Campaign in Brazil.

KAPLAN (UK) How likely is it that in a mass campaign, when millions of people are being vaccinated, all the serious complications will be found or even reported?

VOEGELI (Brazil) I refer to the answer to the first and second question of the former discussant.

FOEGE (USA) Complication rates usually indicate minimum rates because of the difficulty in following vaccinated people. In West Africa it has been impossible to determine complications in tens of millions of persons, therefore we cannot quote useful complication rates from the West African campaign.

HOCHSTEIN-MINTZEL (West Germany) (1) What are the innocuity tests in rabbits and guinea-pigs? (2) Are there tests for adventitious viruses?

VOEGELI (Brazil) (1) Tests on innocuity are performed at the Connaught Medical Research Laboratories, in samples shipped by us, on guinea-pigs (intratesticular reaction and intratesticular transferences). (2) No.

PERKINS (UK) I think that it should be recorded that leucosis-free virus seed material is now used for the production of yellow-fever vaccine which is made in RIF-free eggs. WHO have recently given their approval to the use of such virus seed.

ESPMARK (Sweden) In relation to the question about the possible danger of leucosis virus in South America I want to show a figure on the incidence of leucaemia, lymphome and Hodgkin's disease in the USA and in certain US states. As you see, the nationwide curve for the USA is steadily increasing, but in Texas, where after 1948 practically all smallpox vaccine was of egg origin, the curve has since then been under the state-wide curve. This implies to me that at least egg vaccine does not bring about an increase in the incidence of those malignancies.

EHRENGUT (West Germany) Did you observe vaccinial ulcers following i.c. injections of egg vaccine by the jet-gun? In Puerto Rico they observed 10% of ulcers in the vaccinees.

VOEGELI (Brazil) No, we did not.

SESSION III

LABORATORY TESTING OF SMALLPOX VACCINE

Chairman: Professor W. HENNESSEN (West Germany)
Secretary: Dr R. BROUWER (The Netherlands)

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THE CONTROL OF VACCINE QUALITY IN THE SMALLPOX ERADICATION PROGRAMME

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Assurance of vaccine of good quality is a basic element in the implementation of a successful smallpox eradication programme. We must assure, to the extent possible, that once vaccine has reached the arm of the vaccinee a successful response is obtained, as the opportunity to vaccinate him again may be achieved only with great difficulty and at high cost. As you know, many smallpox endemic areas are difficult geographically. For instance, in Ethiopia 80% of the population live in mountainous areas or desert which can be reached only after one to several days' travel by mule, camel or on foot. In the Asian subcontinent, although communications are relatively good, temperatures of 40–50 °C are not uncommon.

When the smallpox eradication programme was started in 1967 our first concern was to assess the quality of vaccine in use at that time. Since all vaccine donated to the WHO for use in the programme was subject to WHO testing, this provided one measurement of the quality of vaccine in use. The quality of the vaccine was found to be surprisingly poor and large quantities had to be rejected. Only one third of the batches tested at that time met WHO requirements and probably less than 15% of the vaccine then in use in endemic countries was freeze-dried vaccine meeting WHO requirements.

MEASURES FOR THE CONTROL OF VACCINE QUALITY

During 1967 and 1968 several essential measures were undertaken to improve the situation. As a first step it was decided that a simple and practical manual dealing with the methodology of vaccine production should be developed and distributed. Accordingly, in 1968 a vaccine-producers' seminar was held, with the participation of scientists from Canada, Czechoslovakia, the Netherlands, UK, USA and USSR. During this seminar a production manual was produced and subsequently this has been widely distributed.

To increase the capacity for vaccine testing and to augment advisory services a WHO International Reference Centre for Smallpox Vaccine was designated (the Rijks Institute, Netherlands) and a WHO Regional Reference Centre (Connaught Laboratories, Canada). The former deals with vaccines in Asia,

Table I. *Geographical distribution of producers of freeze-dried smallpox vaccine (September 1972)*

WHO region	Number of Producers	
	In routine production	Developing production
Africa	4	2
The Americas	10	2
Eastern Mediterranean	2	5
Europe	26	3
South-East Asia	8	1
Western Pacific	12	1
Total	62	14

Europe and Africa and the latter with South America. With the establishment of these reference centres it was possible to encourage the regular submission of samples from all producers whose vaccine was being employed for the eradication programme. These laboratories, as well as others, have also provided consultant services, and the WHO as well as UNICEF have provided various items of production equipment to producers, particularly those in endemic areas, to augment their production capacity.

TESTING RESULTS

Table I shows the geographical distribution of freeze-dried smallpox-vaccine producers throughout the world in 1972. At present 62 producers are actively engaged in such production. The total amount of vaccine produced is approximately 300 million doses yearly.

A summary of the results obtained in the testing of vaccine batches by the WHO Reference Centres during the last 6 years is shown in Table II. More than 1300 production batches submitted by 43 producers have been tested. The batches tested include those donated to the programme, either through WHO or on a bilateral basis, samples submitted for independent regular testing and samples of experimental batches produced by laboratories during development of production. Although the summary results do not represent samples systematically collected from individual producers, certain general observations can be made.

During 1967 and 1968 only one third to one half of the test batches met WHO requirements in terms of initial potency, heat-stability and bacterial count. However, since 1969 more than three-quarters of all batches submitted have been found to be satisfactory. The somewhat lower proportion of satisfactory batches tested in 1971 and 1972 reflects primarily the fact that during this period more experimental batches were obtained from producers at the developing stage. In addition, in 1972 a large number of vaccine batches prepared on CAM was received. Of 24 batches, only four met heat-stability requirements.

Reasons for the batches of vaccine being designated as unsatisfactory are summarized in Table III. In this Table unsatisfactory batches are tabulated according to the first result which failed to meet requirements. If a batch failed on initial titre it is tabulated

Table II. *Results of vaccine quality control by WHO Reference Centres*

Year	Number of batches tested			Satisfactory (%)
	Total	Satisfactory	Unsatisfactory	
1967	73	23	50	31
1968	169	99	70	58
1969	235	177	58	76
1970	412	336	76	82
1971	233	180	53	77
1972 (Sept.)	225	171	54	76

Table III. *Causes of unsatisfactory batches*

Year	Total tested	Number of batches unsatisfactory				
		Total	Initial titre	Heat-stability	Bacterial count	Other
1967	73	50	47	3	—	—
1968	169	70	36	33	1	—
1969	235	58	33	24	1	—
1970	412	76	34	35	3	4
1971	233	53	32	16	5	—
1972 (Sept.)	225	54	26	27	1	—

under 'unsatisfactory initial titre'; if a batch passed the initial titre but failed heat-stability it is tabulated under 'unsatisfactory heat stability', etc. As can be seen, poor initial potency and heat-stability are the major causes of batches being declared unacceptable. The number of batches found to be satisfactory for heat-stability which failed because of a high bacterial count are consistently few. Four batches in 1970 were rejected for another reason – extremely unsatisfactory solubility.

THREE MAJOR DEVELOPMENTS RELATED TO VACCINE PRODUCTION AND CONTROL

During the last 6 years three major developments of note have occurred which relate to vaccine production and control.

Since 1968 the bifurcated needle, originally developed by the Wyeth Company, USA, has become the recommended tool for multiple puncture vaccination in the programme. The needles have several advantages: empirically this method in the field produces the highest success rate as compared with the multiple-pressure or scarification method; the method of vaccination is so simple that 10 min training is sufficient for anyone who is to be assigned as vaccinator; only a small dose of vaccine is required – 0·0025 ml, a quarter of the usual dose.

Table IV. *Types of vaccinia strains being employed by vaccine producers*

Strains	Africa	Americas	Asia	Europe	Total
		Year: 1968			
Lister	3	3	9	8	23
New York Board of Health	—	5	—	—	5
EM-63	—	—	—	4	4
Institut Pasteur	—	2	3	3	8
Berne	—	—	—	2	2
Patwadangar	—	—	3	—	3
Other*	1	1	7	8	17
Unknown	2	3	3	4	12
Total	6	14	25	29	74
		Year: 1972			
Lister	4	7	22	15	48
New York Board of Health	—	5	—	—	5
EM-63	—	—	—	3	3
Institut Pasteur	—	—	—	3	3
Berne	—	1	—	1	2
Patwadangar	—	—	4	—	4
Other*	1	—	—	7	8
Unknown	1	1	1	—	3
Total	6	14	27	29	76

* Others include Aosta, Bohemia, Bordeaux, Budapest, BM-1, Finland, Hamburg, Ikeda and Massachusetts 999.

However, there was a problem when the bifurcated needles were first introduced. In 1967 and 1968 the amount of vaccine fill in the final containers from various producers ranged widely from 0·2 to 2·0 ml. Using an ampoule containing, for example, 1·0 ml of vaccine, one can perform about 400 vaccinations with the bifurcated needle. However, since vaccinators in many programmes perform only 50–150 vaccinations per day, a substantial wastage of vaccine occurs since it is a matter of policy that reconstituted vaccine be discarded at the end of a working day. All producers were therefore encouraged to produce ampoules or vials containing no more than 0·25 ml vaccine fill. In 1972 all but a very few producers are producing vaccine in ampoules or vials of this capacity. This, of course, results in a considerable economy of vaccine usage as well as a simplification in the operation.

The second change has been in the strain of vaccinia virus used. Table IV shows the vaccinia strains used by different producers in 1968 and 1972. In 1968, 74 producers (including those still at the developing stage) employed six principal strains of vaccinia virus in addition to nine miscellaneous strains. The Lister

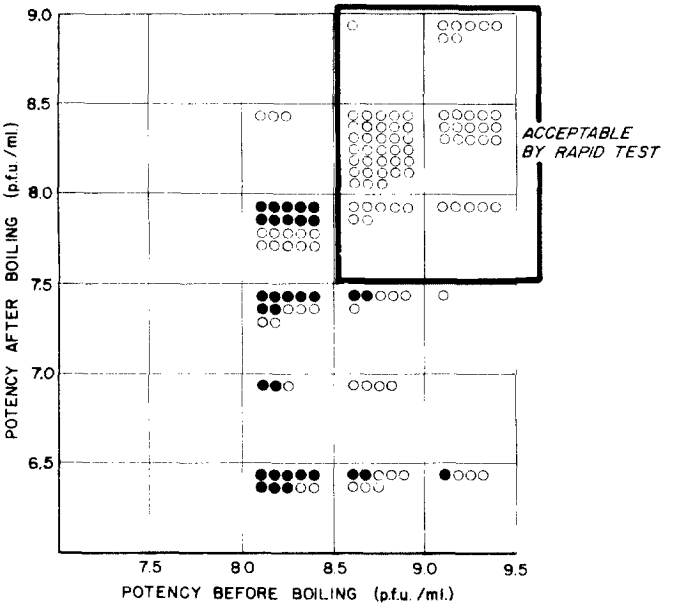

Fig. 1. Rapid screening versus conventional heat-stability test results from 139 batches of smallpox vaccine. ○, Passed conventional heat-stability test; ●, failed conventional heat-stability test.

strain was employed most frequently – by 23 producers (30%). During the last 4 years a marked increase in the use of the Lister strain was noted. In 1972, of 76 producers, 48 (63%) employed this strain. In terms of quantity of production, however, more than 90% of all freeze-dried vaccine produced contains one of these three strains or the Patwadangar strain. To facilitate the work of laboratories in changing strains, the WHO Reference Laboratory produced a quantity of seed virus of the Lister strain which is available on request to any producer who is interested in utilizing this strain.

A third change of interest occurred in the method of heat-stability testing. Conventional heat-stability testing of 4 weeks at 37 °C is inevitably a prolonged process, and if the vaccine is urgently needed this delay may result in an interruption of vaccine flow to the programme. Studies were made of the rapid heat-stability test, which calls for the vaccine to be heated to 100 °C for 1 h to determine if the results obtained could be correlated with those obtained by the conventional heat-stability test. In 1969 in co-operation with the WHO International Reference Centre, comparisons were made of batches of vaccine subjected to the two tests.

As shown in Fig. 1, it was found that vaccine which contained over 8·5 pfu/ml on initial potency and mantained a titre of more than 7·5 pfu/ml after boiling consistently met the standard requirements for heat-stability. Accordingly, since 1969 the testing procedures at the WHO Reference Laboratory have been modified. Vaccine samples are first tested for stability by incubation at 100 °C for 1 h. If the initial potency is above 8·5 pfu/ml and

Table V. *Results of rapid screening test for heat-stability*

Year	Lots tested	Passed rapid test	Failed rapid test but passed conventional test	Failed both tests
1969	25	19	3	3
1970	151	111	22	18
1971	91	52	33	6
1972 (Sept.)	70	42	21	7
Total	337	224	79	34

the potency after boiling above 7·5 pfu/ml, the vaccine is regarded as acceptable. Vaccine which fails to pass this screening test is further tested by the conventional heat-stability test for final determination of its stability.

This approach has simplified vaccine-testing procedures. Table V shows the results since this method has been employed. Since 1969, 337 batches have been tested with this method by the WHO Reference Laboratory, and of these, 224 batches (67 %) were determined to be acceptable by the rapid test. During this period it has become apparent that, for reasons unknown, vaccine prepared in vials as well as vaccines from certain producers consistently undergo a large reduction in potency when subjected to a temperature of 100 °C for 1 h. Vaccines from these producers, therefore, are selectively tested only by the conventional heat-stability method.

SPECIAL REVIEW OF VACCINE QUALITY

Lastly, as shown in Table VI, I would like to summarize briefly data regarding the quality of vaccine from 15 producers whose vaccine is currently being used in countries which are now, or recently were, endemic. These countries include Brazil, the African countries south of the Sahara, Indonesia and presently endemic countries on the Asian subcontinent.

These areas are, of course, the most important so far as the implementation of the global eradication programme is concerned. It is estimated that the quantity of vaccine manufactured during the last 12 months by the producers concerned was approximately 240 million doses. Ten of the 15 producers are located in developing countries. The average titres of the most recently tested five successive lots submitted to the WHO Reference Centres are shown in Table VI. All except producer 15 produced vaccine with an initial potency ranging from 8·3 to 9·2 pfu/ml. Vaccine manufactured by producers 1–9 showed acceptable heat-stability by the rapid screening test and vaccine from producers 10–14 showed acceptable stability by conventional heat-stability testing. Of 15 producers, 11 produced vaccine of which the bacterial content per ml was less than 20. No pathogens were detected. Taking into account the quantities of vaccine produced by the different producers, it may be stated that over 95 % of the vaccine (excepting that of producer 15) used in these essential programmes meets WHO requirements.

Clearly, these vaccines are performing well under field conditions, as routine assessment results from all programmes almost invariably show primary take

Table VI. *Vaccine quality being used in Brazil, sub-Saharan African continent, Indonesia and endemic countries of the Asian subcontinent*

Producer's number	Initial titre	Titre after heating	Bacterial count/ml.
		(After 1 h at 100 °C)	
1	9·2	8·1	< 10
2	9·0	8·0	15
3	8·9	8·0	70
4	8·8	7·8	30
5	8·8	7·5	< 10
6	8·7	8·0	< 10
7	8·7	7·5	50
8	8·6	8·0	< 10
9	8·4	7·6	< 10
		(After 4 weeks at 37 °C)	
10	8·9	8·6	0
11	8·7	8·3	< 10
12	8·7	8·2	150
13	8·7	8·0	< 10
14	8·3	7·9	20
15†	7·9	7·4	< 10

* Average titre (pfu/ml) of the latest five lots submitted to WHO Reference Centres.
† Vaccine produced on CAM and used in a country which is no longer endemic.

Table VII. *Testing results of field vaccine samples*

Year	Country	Number of samples tested	Titre (pfu/ml)			
			< 7·6	7·7–7·9	8·0–8·5	> 8·6
1968	Indonesia	21	1	2	4	14
1969	Indonesia	21	0	0	0	21
	Nepal	20	5	3	12	0
1970	Brazil	11	0	2	9	0
	Burma	14	0	3	10	1
1971	India (Gujarat)	9	0	0	7	2
	India (Bihar)	8	1	0	2	5

rates exceeding 95%. Limited information is also available regarding titres of vaccine samples obtained from the field during assessment. As can be seen in Table VII, the results have generally been satisfactory.

CONCLUSION

In brief, a great deal of improvement has been made in ensuring the supply of good-quality vaccine to the eradication programme. Although the extent and frequency of testing is still not what we believe it should be, the control scheme seems to have worked quite well, owing to the excellent co-operation of the producers and the WHO Reference Laboratories. As already observed, the eradication programme is proceeding satisfactorily and rapidly, circumscribing the endemic areas to more and more limited areas. However, even if a nil incidence is reached, the WHO Expert Committee recommended that at least 2 years of active surveillance will be required to confirm the absence of smallpox in countries where smallpox was once endemic. The vaccination programme must continue, especially in high-risk areas and high-risk populations. Therefore due attention must continue to be paid to the control of vaccine quality, and continuing supplies of vaccine will, of course, be required.

SUMMARY

The assurance of freeze-dried smallpox vaccine of good quality is a basic element in the implementation of a successful eradication programme. In 1967, when the programme started, the quality of the freeze-dried vaccine was not satisfactory; two thirds of the batches did not meet requirements when tested by WHO testing laboratories. Hence, in 1967 and 1968 several measures were undertaken to improve the situation. From 1967 to 1972 over 1300 batches were received from producers throughout the world and tested by WHO Reference Centres. During the last three years over three-quarters of the batches tested met WHO requirements. It is estimated that, to date, 95% of the freeze-dried vaccine being used in the important areas for the eradication effort is of the required standard.

We particularly express our appreciation of the generous co-operation and dedication of the WHO Reference Laboratories in Utrecht and Toronto, since without such co-operation the vaccine control scheme could not have been carried out, and we all know that the job of the assay laboratory is not an easy one.

Discussion

POLAK (The Netherlands) A number of unsatisfactory batches presumably failed to pass two or three tests. Double and triple counts, however, are not accounted for in Table III. It looks as if the results of the three tests served sequentially as criteria for acceptance or rejection; the initial titre first, the bacterial count last. If so, the low frequency of high bacterial counts is an underestimate. Were the test results applied in the same serial order for all batches?

ARITA (WHO) This table has been prepared in this way for the sake of simplicity. The low frequency of high bacterial counts is not an underestimate because all tests were done on all batches of vaccine even when the initial potency was unsatisfactory.

BERNSTEIN (USA) (Suggestion to laboratories testing vaccine from different sources.) I have interpreted the proposal of the accelerated stability test (i.e. 100 °C for 1 h or 37 °C for 4 weeks) to be an attempt to predict the stability of the vaccine stored at 2–8 °C. Attempts have been made to establish such correlations for other vaccines with little success. Since the WHO laboratories have access to lots of vaccine that do not pass the accelerated stability tests, it would be of interest to set some of these lots aside and follow their stability characteristics during storage at 2–8 °C.

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REFLEXIONS ON THE CONTROL, TESTING PROCEDURES AND ACCEPTANCE CRITERIA FOR SMALLPOX VACCINE

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For 150 years smallpox vaccine has been the cornerstone of public-health measures directed against infectious diseases and it is its efficacy to which the gradual conquest of clinical smallpox in the world is largely attributable. The various complications which may follow its use were well known and tacitly accepted as the price one had to pay for freedom from smallpox in the community(**2**, **11**, **12**).

It has been accepted that some types of complications, and we might call them 'specific', are consequences of a particular and rather exceptional type of host–virus relationship, the occurrence of which can be substantially reduced by careful scrutinization of persons to be vaccinated, including potential contacts in their families. Thus the reduction of these complications is beyond the control of the vaccine producer and it is attainable solely by strictly applying the list of contraindications.

It seems that until recently studies and observations have been directed more towards immunological aspects, towards conditions ensuring successful vaccinations and towards establishing the degree and duration of protection after vaccination, than towards studies aiming at increasing the margin of safety of the vaccine itself.

The reason for such an attitude was partly the concern of public-health people to be ensured of operating with a sufficiently potent and stable vaccine, in lack of which all efforts in their immunization programmes would be wasted – partly because it was notorious that some complications of 'specific' nature are unavoidable; and partly because serious complications of a 'non-specific' type, due to faulty vaccine production, seldom appeared and even more seldom received full documentation.

However, by scrutinizing the vast literature on smallpox vaccination one finds numerous descriptions of individual cases or even outbreaks of tetanus, streptococcal and staphylococcal sepsis and local diphtheritic infection. It seems that in many cases the origin of these complications could be justifiably traced to the particular vaccine batches used, whereas in other cases the infection could have been of secondary or even coincidental nature.

Nevertheless these incidents, some of which were described quite recently, must serve to the vaccine producers as a constant warning that despite the improved production methods, persistent alertness is needed to ensure purity of the produced vaccine lots.

By perusing pharmacopoeias and other sets of regulations of various countries (Canada, Britain, USA, France), edited between the wars, it is interesting to note how few changes were introduced in the requirements which controlled the production of smallpox

vaccine. The Minimum Requirements as laid down in these documents were below what could have been attained by techniques already available at that time. This is particularly true with regard to the purity of the vaccine. The permissible limit of contaminants was set at 1000/ml, a number which figured in most regulations until not long ago and which figures even nowadays in some. Other standards were expressed in rather indefinite terms, particularly the one requesting 'freedom of pathogens'.

The potency standards, although lacking the quantitative criteria of nowadays, should have been sufficient to achieve satisfactory take-rates in primary vaccination. On the other hand, stability requirements were understandably non-existent, since only storage at low temperatures has ensured the usefulness of the vaccine even for a short time. The lack of precision in the definition of standards caused both vaccine producers and control organs to make decisions which were based more frequently on arbitrariness than on rules. All this resulted too often in frustration for physicians and epidemiologists, who discovered too late that they were using substandard vaccines.

The WHO and its Expert Committee should receive most of the credit for the changes and initiatives which have been introduced in this field within about the last 15 years. This impetus caused not only the development of defined testing procedures and standards, but also generated the need for the development of new preparation methods, the result of which is the pure, potent, and stable freeze-dried vaccine which so greatly surpasses its older brother, the liquid, glycerinated smallpox vaccine.

The requirements for smallpox vaccine as laid down by the WHO in 1959 in the form of recommendations, marked the first important step towards reaching an agreement among the many vaccine-producing institutes regarding attainable and applicable standards.

Although these requirements were already regarded at that time as indeed minimal, nevertheless they embraced all aspects that needed to be tested and standardized. Further, since standards are meaningful and comparable only if they are based on standardized testing procedures, such procedures became part of the requirements.

As a side-effect, it also became obvious to what degree the national requirements of most countries were lacking in precision and comprehensiveness.

The concept of an expert group of the WHO drawing up international recommendations regarding requirements for a biological substance was further vindicated by the readiness and speed by which these regulations became accepted as guidelines by most national control authorities. This is particularly true for those countries that were willing to participate in an international exchange of biological substances, including offering assistance in immunization programmes of the WHO.

The first requirements published in 1959 were amended in 1965(**7**, **8**). This modified version is in effect at the present time, although in 1968 a study group of the WHO put forward recommendations to make some of the testing methods and standards more stringent. However, these recommendations never reached the stage of official publication(**13**).

The changes introduced into the modified version of requirements covered most aspects of vaccine production.

A practical system of establishing primary and secondary seed lots was recommended. It is to be hoped that this very useful principle has been adopted by all vaccine producers. In a future edition it might be desirable to stress the need for bacterial sterility of the seed lots, and for proof of absence of contaminating viruses(**6**). Perhaps the WHO could take steps for the implementation of conclusions originating from some excellent studies revealing the nature and genetic properties of a number of vaccinia virus strains used for vaccine production throughout the world. Some of these viral strains were shown to have superior qualities with regard to reactogenicity and homogeneity(**1, 4, 5**). A great service would be rendered if preparation and distribution of primary seed virus became an established practice of one of the Reference Centres. The amended version reduced the permissible number of contaminants from 1000 to 500 per ml. This is still far too high a number; one tenth of it, namely 50 contaminants per ml of the filled vaccine, would be much more in accordance with the level of contaminants in presently produced vaccine lots. Such stiffening of the requirement would at the same time facilitate the attainment of other safety standards. It may well be that a dilution method which determines the most probable number of contaminants is superior to the poured plate method, because the former is performed in such a way as to ensure the growth of both aerobes and anaerobes.

The tests and requirements regarding freedom from pathogens, notably haemolytic streptococci, coagulase-positive staphylococci and *B. coli*, are far too lenient. It is required that 0·03 ml be tested and shown to be free from the above bacteria. The newest regulations of the NIH in Washington require that the absence of pyogenous cocci must be demonstrated in a 3 ml sample of the final bulk, which is 100 times larger than the sample recommended by the WHO; and the absence of *B. coli* must be proved in liquid medium using a 5 ml sample of the vaccine, which is 170 times the amount requested by the WHO.

As long as smallpox vaccine is being produced from infected animal skin, a great many safety measures must be focused on the possible contamination of the viral harvest with anaerobes. This is not because of frequent incidents in vaccinated persons. Fortunately the technique and nature of smallpox vaccination usually does not render favourable conditions for an anaerobic infection. However, when carefully and persistently tested, anaerobic bacteria can occasionally be found in batches of smallpox vaccine.

When dealing with this problem three questions arise. (1) At what stage of vaccine production should the test for anaerobes be performed? (2) Are the present tests for anaerobes satisfactory? (3) Should the presence of non-pathogenic anaerobes affect the acceptance of a vaccine lot?

Answering the first question it should be considered whether or not the test for anaerobes should be performed on the unprocessed viral harvest, rather than on the final bulk, as recommended in the WHO requirements. The presence of pathogenic anaerobes in the viral pulp should make the latter unsuitable for

further processing, regardless of whether or not anaerobes are demonstrated in the final bulk. The presently described test for anaerobes may not be quite adequate, partly because of the small amount required to be tested – 1 ml of the final bulk – and partly because it relies solely on heated samples for the isolation of anaerobes. It should be taken into account that a large group of pathogenic clostridia – the perfringens group, which is so ubiquitous in any material of animal origin – does not sporulate readily, and since in the process of heating the vegetative forms are eliminated, one may end up with falsely negative test results. The introduction of the anaerobic jar among the testing tools would give more assurance regarding the purity of the product.

Finally, when considering the safety of the vaccine there seems to be little reason for making distinction between aerobes and anaerobes, provided non-pathogenicity has been proved.

The revised version of the WHO requirements introduced a compulsory innocuity test on the final bulk, although with very little specification. In a new edition details of such a test should be given.

In the 1950s and 1960s a large number of well-designed studies was undertaken to determine the factors which render the vaccine potent and stable and to design the necessary tests by which to prove that the requested criteria are being met. Presently there is hardly any argument about potency and stability criteria, nor about the related testing methods. Pock counting on the chorioallantoic membrane has been generally accepted as the most relevant and consistent test. There might be a difference in opinion regarding some technical details, like the length of egg incubation and the size of inoculum, or how to interpret marginal results or, whether or not to apply confidence limits for their evaluation. All the elements necessary for consistent test results were recently summarized in an elaborate study and published as a working document of the WHO(3).

However, some people might prefer the recently published NIH Requirements according to which the potency and stability of the test vaccine is expressed in terms relative to a reference preparation.

The newest US regulations, which were in the making since 1967, replaced those which were in effect for more than 20 years(**10**). The difference between the two sets is quite profound. The safety rules are by far more stringent than those laid down in the previous set or by the WHO. Potency requirements are identical to those of the WHO, except that a reference vaccine is an inherent part of the test. It is only the stability criterion where unnecessary leniency has been shown. These regulations incorporate also the criteria for jet vaccine, something which has been omitted so far from WHO documents, except for a non-committal passage in a report on smallpox eradication(**9**).

The jet vaccine, according to the NIH, shall contain not more than 1 non-pathogenic organism per 100 doses, i.e. per 10 ml of the reconstituted vaccine. This is a requirement which comes close to the sterility standards of parenteral drugs. And this is probably what the next set of regulations will request.

The minimum potency of the jet vaccine has been set at $10^{5\cdot5}$ pock forming units per single dose, although this value is also being expressed relative to the Reference Vaccine.

CONCLUSIONS

The expert committee on smallpox of the WHO has provided in the last few years excellent guidelines which greatly contribute towards the perfecting of smallpox vaccine. However, it appears that the standards as laid down a few years ago are being surpassed by the present production methods. There seems to be a need to bring these standards up to date and to correlate them to vaccine qualities which are readily attainable.

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Discussion

MAJER (Switzerland) There is a little confusion and inconsistency in the interpretation of the WHO requirements for smallpox vaccine. I think that these requirements are not only made for the eradication programme but for all countries. If it is stated that the freeze-dried vaccine should be stored at $+4\,^{\circ}C$ I do not think that it is necessary to introduce a stability test only because in the eradication programme in a few countries these storage requirements cannot be fulfilled. It is out of discussion that the titre of the vaccine should be $\geqslant {}^{10}\log 8{\cdot}0$, but to this requirement should be added that 'Only if

these storage requirements cannot be fulfilled one should make sure (by heating during 4 weeks at 37 °C) that the vaccine is stable.'

ARITA (WHO) I am sorry but I do not see the confusion.

HARBOE (Norway) Dr Majer means that in a country like ours all the users of the vaccine have refrigeration facilities and the shipment is done conveniently within one or two days. Under these circumstances heat-stability tests make no sense. However, we might get into great difficulties in case we purify the vaccine and cannot make it as stable as is required for hot countries. In that case we would simply say that some vaccine is prepared for the tropics and other vaccine is prepared for more temperate climates.

COHEN (The Netherlands). The aim of the requirements for vaccines is the stimulation of international exchange of material. It is free for each national control authority to make his own specifications. The only thing you cannot claim in that case is that this vaccine meets WHO requirements and if it is brought to another country it is up to the local authorities there whether they will accept it or not.

EDSALL (UK) In considering the proposal that new, revised and doubtless more stringent requirements for smallpox vaccine be developed, I would like to call attention to the general tendency, in drafting or revising requirements, to lean on laboratory tests and on easily quantitated tests, for assurance. Inevitably many such requirements may well be determined by administrative decision rather than on a sound logical basis; on the other hand, most such requirements do not include regular human testing which sometimes yields information not obtainable by the laboratory tests required. Therefore I would like to urge that the IABS give its attention to revision of the smallpox vaccine requirements, but that in doing so it give close attention to the realism and the relevance of such requirements.

MURRAY (UK) I would like to comment that I applaud Dr Fenje's plea for more rigorous quality control in the production of conventional smallpox vaccine. With regard to bacterial content in particular, I should consider anything short of bacterial sterility to be only a compromise. Since it is possible to prepare a bacteriologically sterile vaccine in cell cultures I would suggest that the production of such a vaccine ought to be our aim.

EVANS (UK) I would like to comment that if the WHO requirements for smallpox vaccine are to be improved as suggested by Dr Fenje, the improvements should be realistic, bearing in mind that such improvements are not going to jeopardize the supply of vaccine throughout the world.

NETTER (France) I want to comment on residual moisture because I am not sure that titration of residual moisture is a more reproducible test than accelerated storage test. However, residual moisture content is dependent on the quality of stabilizers used for freeze-drying.

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POTENCY TESTING OF SMALLPOX VACCINES BY THE CHORIOALLANTOIC MEMBRANE ASSAY

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Control of the potency of smallpox vaccine requires that the assay method be precise, reproducible and correlate with effective immunization in man.

The rabbit scarification method was the required laboratory test system for the determination of the potency of smallpox vaccines in the United States until June 1971. At that time, the United States Public Health Service Regulations were changed to permit licensed manufacturers to select either the chorioallantoic membrane (CAM) assay or the rabbit scarification method for determining the vaccine potency.

Control of the potency of smallpox vaccine in the United States is based on the use of a national reference vaccine preparation having a potency equivalent to that of the international Reference Preparation of Smallpox Vaccine. Manufacturers are required to test each lot of vaccine in parallel with the US reference vaccine. Use of this reference in every test provides a means for determining the acceptability of each test. A smallpox vaccine intended for multiple pressure administration must have a potency at least equivalent to that obtained for the US Reference Smallpox Vaccine, Lot 2, when tested in parallel.

Recently, Slonim *et al.*(3) have shown that the volume of the inoculum used in the CAM test can affect the results of the test in such a manner that the greater the volume, the smaller the number of pocks on the CAM. Therefore a study was made of the effect of using two different volumes of inoculum (0·1 and 0·2 ml) on the potency determination of the US Reference Smallpox Vaccine. The volumes selected are those generally reported in studies using the CAM assay.

MATERIALS AND METHODS

The CAM procedure used in our laboratory was recently published(1) and is similar to that reported by the World Health Organization(4).

The US Reference Vaccine, Lot 2, was reconstituted with 3 ml beef heart infusion broth. Further dilutions were prepared in the same medium. The vaccine is tested immediately upon rehydration.

Dried commercial vaccines were reconstituted according to the directions provided by the manufacturers, and subsequent dilutions were prepared in beef heart infusion broth. Through experience we have found a 1 ml volume to be an appropriate sample size for preparing the initial dilution of reconstituted vaccines.

Table I. *Acceptance limits of CAM assays of US Reference smallpox Vaccine, Lot 2**

Number of tests	99 % Level of significance			
	0·2 ml inoculum		0·1 ml inoculum	
	Lower	Upper	Lower	Upper
1	7·6	8·6	7·9	8·6
2	7·8	8·4	8·0	8·4
3	7·8	8·4	8·0	8·4
4	7·8	8·3	8·0	8·4
5	7·9	8·3	8·0	8·4

* ^{10}log/ml.

Although we would prefer to use the same volume for liquid vaccines to prepare the initial dilution, the tedious process of removing the vaccine from a large number of capillaries would increase the chance of contaminating the product. Thus, we start with a 0·5 ml volume when testing liquid vaccines.

For testing the potency of commercial vaccine we use the dilutions $10^{-5\cdot5}$ and $10^{-6\cdot0}$. This dilution range is wide enough to test vaccines of all US licensed manufacturers.

For the reference vaccine, we inoculate the CAM with $10^{-5\cdot8}$ and $10^{-6\cdot0}$ dilutions.

The number of pocks forming on a single membrane will usually be 10–60, depending upon the dilution and the volume of inoculum.

RESULTS

An analysis of our data for 269 CAM titrations of the US reference using 0·2 ml inoculum showed the population log mean to be 8·1. In order to determine the range of titre for the reference that would indicate the particular test is 'in control', further analysis of the data showed the mean titre, $10^{8\cdot1}$ pfu/ml, to have a standard deviation of $\pm 0\cdot2$ log. The limits of acceptance for single and replicate tests were developed and are shown in Table I.

Thus, for the reference vaccine the lower limit for a satisfactory test is $10^{7\cdot6}$ pfu/ml when 0·2 ml is used as inoculum. In the same test, the lowest acceptable titre for an unknown vaccine would also be $10^{7\cdot6}$ pfu/ml. If a value of less than $10^{7\cdot6}$ pfu/ml is obtained for the reference the result of this test is not acceptable and the test must be repeated regardless of the titre obtained for the unknown vaccine. The average of the titres obtained in the original test and in the repeat test must not be less than $10^{7\cdot8}$.

Based on the results of 55 tests on the reference in which 0·1 ml inoculum was used, the mean titre was determined to be $10^{8\cdot2}$ pfu/ml with a standard deviation of $\pm 0\cdot1$ log. A statistical analysis of the data for 0·1 ml inoculum showed that at the 99 % level of significance, the acceptable value of a single test is $10^{8\cdot2}$ pfu $\pm 0\cdot3$ log as shown in Table I. The lower limit for a satisfactory vaccine is $10^{7\cdot9}$ pfu/ml when 0·1 ml inoculum is used.

Table II. *Comparison of potency results for US Reference Smallpox Vaccine, Lot 2, between laboratories*

Manufacturer	Inoculum	Pock-forming units (log_{10}/ml)	
		Manufacturer	BB
A	0·2 ml	8·1 (7·8–8·2)*	8·1 (7·9–8·2)
B	0·1 ml.	8·3 (8·1–8·5)	8·2 (8·2–8·3)

* Range.

Table III. *Comparison of potency results for commercial smallpox vaccines between laboratories*

Manufacturer	Inoculum	Type of vaccine	Pock-forming units (log_{10}/ml)	
			Manufacturer	BB
A	0·2 ml.	Dried	8·0 (7·8–8·2)*	7·9 (7·8–8·0)
B	0·1 ml.	Dried	8·2 (8·1–8·3)	8·1 (7·9–8·2)
A	0·2 ml.	Liquid	8·0 (7·7–8·3)	7·8 (7·7–7·9)

* Range.

Another criterion for a satisfactory test is the requirement that for each dilution used at least three membranes of each group of five membranes inoculated must yield satisfactory data. The expected number of pocks, 10–60 per membrane, must be distributed in such a manner that they can be counted. On occasion a membrane may contain pocks which overlap or are confluent in such a manner that a count cannot be taken. Similarly, a membrane may be 'lost' due to the death of the embryo during the test period. These results must also be recorded.

The request of three manufacturers to use the CAM assay has been approved. However, since receiving approval, only two manufacturers have submitted lots of smallpox vaccine to the Bureau of Biologics for testing.

There is excellent agreement of potency results between these laboratories and the Bureau for the U.S. Reference as displayed in Table II. The need to report the inoculum used in determining the potency of a reference is apparent when we compare the value obtained by the Bureau for the reference.

A summary of our experience in testing manufacturers' vaccines by the CAM method is displayed in Table III.

DISCUSSION

Before a manufacturer is permitted to use the CAM assay in the determination of the potency of his vaccine, he must submit to the Bureau, for approval, data relative to his experience with the US Reference Vaccine, Lot 2. The data submitted must include the mean titre, standard deviation, confidence limits and details of his test method.

Because the source of embryonated eggs can influence both the mean titre and variability of the test the acceptable limit values obtained by the manufacturer are used for evaluating the manufacturers' test results, and the values determined by the Bureau are used for evaluating all tests performed by the Bureau.

Although Slonim *et al.* recommended using an inoculum of 0·025 ml on the CAM, we found it difficult and time consuming to consistently measure such a small volume accurately. Therefore, this study was limited to the two volumes, 0·1 ml and 0·2 ml, generally reported in studies using the CAM assay.

A review of our data for individual vaccine tests and those data submitted by the respective manufacturer has shown that 100% of the potency values obtained between laboratories are in agreement with a maximum difference of $\pm 0{\cdot}3$ log for the reference vaccine and $+0{\cdot}4$ log for the manufacturers' vaccines.

The potency of the U.S. Reference Vaccine and vaccines produced by US licensed manufacturers is of the magnitude adopted by the WHO as necessary for smallpox vaccination in human subjects (**4**). The US reference has been used successfully in the vaccination of susceptible children (**2**).

It is apparent from the data reported here that the CAM assay is reliable, reproducible and applicable to the control potency testing of dried and liquid smallpox vaccines produced by US licensed manufacturers when used within the limits discussed.

SUMMARY

The chorioallantoic membrane (CAM) assay has been shown to be a reliable and reproducible method for determining the potency of smallpox vaccines produced by manufacturers licensed by the United States. The CAM test results are 100% in agreement between the manufacturers and the Bureau of Biologics for the US Reference Smallpox Vaccine and dried and liquid vaccines produced by U.S. licensed manufacturers.

The potency of the U.S. Reference Smallpox Vaccine and vaccines produced by US licensed manufacturers is of the magnitude adopted by the WHO as necessary for smallpox vaccination in human subjects.

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THE POTENCY ASSAY OF SMALLPOX VACCINES PREPARED IN CELL CULTURES

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During the development of a tissue-culture smallpox vaccine at Elstree differences between infectivity assays in chorioallantoic membranes and in cell cultures were observed. In the collaborative assay of the international reference preparation of smallpox vaccine, marked discrepancies were found in the relative potencies of certain of the test vaccines when they were determined in tissue culture and in other substrates (**6**). Though a vaccine derived from cell cultures was not included in the collaborative assay, it was noted that the proposed reference vaccine was unlikely to serve as a useful reference for all vaccines. With the advent of smallpox vaccines prepared in cell cultures, it was considered apposite to examine the validity of the international reference preparation and the influence of the assay substrate in the assay of tissue culture vaccines.

MATERIALS AND METHODS

The cell cultures used both for plaque and quantal assays and for the serial propagation of the Elstree strain were the continuous line cells BSC 1 (monkey), BHK 21 (hamster), RK 13 (rabbit), the human embryo lung diploid cell strain WI 38, and primary cultures of chick embryo (CEC) and rabbit kidney cells (PRK).

A freeze-dried Lister smallpox vaccine derived from sheep skin was used as the reference preparation in all the assays. In some experiments the Elstree strain adapted to rabbit skin by 80 passages was used. Unless otherwise stated, the complete harvest from the cultures, i.e. cells plus supernatant, was taken. All liquid virus suspensions were stored at −70 °C. After thawing, the suspensions were exposed to ultrasonic vibrations of 18–22 kHz for 20 sec.

Quantal assays were done by a cytopathic end-point micro-technique. For quantitative assays pock counts were made in the chick chorioallantois (**8**) and plaque counts were done by conventional methods in monolayer cell cultures. Relative potencies were calculated by standard statistical methods for parallel line assays (**5**) estimating a common slope for all preparations in each assay. For quantitative assays log plaque or pock count was related to log dose and for quantal assays the probit of percentage cytopathic effect was used as response. In addition, a log dose probit line was fitted to data for each preparation separately and titres estimated as the log dose equivalent to a probit of 5.

Table I. *Log potency ratios (M) of supernatants of different vaccinia-infected cell cultures assayed by a quantal technique in BSC 1 and RK 13 cells*

Growth substrate	Assay substrate	Passage level 1	2	3	4	5
BHK 21	BSC 1	−2·64	−2·97	−2·40	−2·60	—
	RK 13	−2·12	−3·25	−3·28	−3·01	—
WI 38	BSC 1	−2·51	−2·58	−1·71	−2·63	−1·97
	RK 13	−2·31	−2·25	−2·69	−2·73	−2·33
BSC 1	BSC 1	−3·34	−2·71	−1·98	−3·91	−2·37
	RK 13	−3·08	−3·26	−3·10	−3·45	−3·14
RK 13	BSC 1	−2·53	−1·56	−2·16	−3·83	−2·90
	RK 13	−2·05	−2·30	−3·22	−3·45	−3·20
CEC	BSC 1	−2·14	−0·89	−1·83	−1·92	−1·39
	RK 13	−1·87	−1·72	−1·80	−2·50	−1·49

Table II. *Log potencies (TCD 50/ml) of supernatants of different vaccinia-infected cell cultures assayed by a quantal technique*

Growth substrate	Assay substrate	Passage level 1	2	3	4	5
BHK 21	BSC 1	3·77	3·32	—	4·38	3·96
	RK 13	4·20	—	3·93	—	—
WI 38	BSC 1	—	3·93	4·57	4·55	—
	RK 13	3·99	4·43	—	4·44	5·11
BSC 1	BSC 1	—	3·82	4·32	—	4·61
	RK 13	—	3·79	4·05	3·76	—
RK 13	BSC 1	3·95	4·94	4·10	3·41	4·06
	RK 13	4·24	—	3·99	3·76	4·24
CEC	BSC 1	4·38	5·38	5·35	5·05	—
	RK 13	4·43	5·49	5·37	4·93	5·05

RESULTS

In quantal assays in cell cultures, the infectivity of the supernatants remained constant during five sequential passages of the Elstree vaccinia virus strain in the different cells. Both log potency ratios against a sheep reference vaccine and TCD 50 titres were of the same magnitude, and BSC 1 and RK 13 cells appeared equally satisfactory as assay substrates (Tables I, II). Infected culture harvests from the seventh passage in RK 13 and primary chick cell cultures and the 80th passage in rabbit skin gave comparable log potencies in BSC 1, RK 13 and

Table III. *Log potencies of the Elstree vaccinia virus strain grown and assayed by a quantal technique in different substrates*

Growth substrate	Expression of potency	Assay substrate		
		BSC 1	CEC	RK 13
BSC 1, pass 10	M	—	—	—
	TCD 50/ml	5·75	5·55	5·75
CEC, pass 7	M	−0·83	−1·11	−1·83
	TCD 50/ml	5·10	5·64	5·66
RK 13, pass 7	M	−2·02	−2·94	−2·82
	TCD 50/ml	4·55	—	4·44
Rabbit skin, pass 80	M	1·36	1·14	1·68
	TCD 50/ml	8·25	8·06	8·49

Table IV. *Log potencies of the Elstree vaccinia virus strain grown and assayed by a quantitative technique in different substrates*

Growth substrate	Assay substrate		
	CAM	CEC	PRK
CEC, pass 7	−2·36	−1·66	−0·82
PRK, pass 1	−0·60	−0·06	0·60
Rabbit skin, pass 80	−0·15	0·78	2·15
Sheep skin	−0·37	—	−0·59

Table V. *Log potency ratios of* 1:3 *dilutions of viral preparations grown in different cells assayed against the undiluted preparations in different substrates*

Growth substrates	Assay substrate		
	CAM	CEC	PRK
Sheep skin	0·37	—	0·59
CEC, pass 10	0·43	—	0·66
RK 13, pass 9	0·54	0·29	0·48
Rabbit skin, pass 80	0·42	0·51	0·80

primary chick cells (Table III). However, in quantitative assays from plaque counts in the different cell cultures and pock counts in chorioallantoic membranes, significantly different log. potency ratios were obtained. It can be seen from Table IV that the substrates used for propagation markedly influenced the results obtained. These differences do not appear to be inherent in the assay system, since a 1:3 dilution of the sheep reference preparation gave the

predicted potency when compared with the undiluted preparation in chorioallantoic membranes and primary rabbit kidney cells (Table IV). Similarly 1:3 dilutions of virus grown in chick and RK13 cell cultures and in rabbit skin gave log potency ratios approximating to 0·5 when compared with the undiluted virus preparations in chorioallantoic membranes and chick and primary rabbit kidney assay substrates (Table V).

DISCUSSION

The relatively uniform susceptibility of the chick chorioallantois to infection with vaccinia virus enables meaningful infectivity assays of smallpox vaccine to be done without a reference preparation. Quantal assays with standardized technique have been used successfully in the routine potency assay of egg-derived smallpox vaccine(**1**). In these studies a correlation was established between assay titres and virus infectivity in man(**2**, **3**, **4**). Although many of the factors influencing the plaque count in quantitative vaccinia virus assays in cell cultures have been elucidated(**7**), variations in the sensitivity of the technique from one laboratory to another preclude its direct application to the potency assay of smallpox vaccine unless a suitable reference vaccine is used. From the work reported here, it is clear that discrepant results may be obtained with virus grown in cell cultures when assayed against a sheep-derived reference vaccine. One may expect to get the predicted potency ratio, irrespective of the assay substrate, provided that the reference preparation is derived from the same source of material. This can be seen in Table V, where dilutions of virus propagated in different cells were assayed in different substrates. It is suggested that the international reference preparation of smallpox vaccine may not be a suitable reference preparation for the potency assay of vaccines prepared in cell cultures, and it might be more appropriate if the reference preparation were derived from the same cells.

SUMMARY

The influences of assay substrate and origin of the reference preparation in the potency assay of smallpox vaccine were studied. In the quantitative assays determined by pock and plaque counts discrepancies in relative potencies with respect to a sheep-derived reference preparation analogous to the international reference preparation of smallpox vaccine were found. It is suggested that the international reference preparation may not be a valid standard for the assay of some smallpox vaccines prepared in cell cultures.

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Session III

GENERAL DISCUSSION

ESPMARK (Sweden) I got the impression from the previous two papers that the diverse results with tissue culture titrations on the whole were less satisfactory than the CAM test. I tried to get the tissue-culture method in the WHO recommendations. I have the feeling that Dr Murray's contribution has further cooled down the WHO committee on this point.

VALLE (Finland) I agree with Dr Murray that in the near future when tissue culture vaccines are going to be accepted for general use, a new international reference preparation, more suitable for the control of this type of vaccine, is needed. The need for a potency test done in tissue cultures is evident. As has been shown, the pock counts on the CAM does not follow the Poissonity. However, the plaque counts do so. Therefore more accurate results and a better reproducibility could be obtained by a plaque assay method. It would also be easier to do large titration series in tissue cultures than in eggs.

HENDERSON (WHO) The potency of the vaccine produced in the USA is right on the border of acceptability and one would expect that if this is incubated for 4 weeks at 37 °C there would be a loss of at least 0·2 or 0·3 logs, in which case the vaccine would not meet WHO requirements. What are the US standards in regard to stability of a vaccine and what are the results being found with these lots you are testing?

FULLER (USA) We have never tested the reference vaccine after 4 weeks at 37 °C. On stability testing of commercial vaccines there is a loss of usually 0·3 logs and never more than 0·6 logs.

HENDERSON (WHO) May I continue in the light of this and in view of Dr Evans' comment earlier about reality and relevance in regard to biologic standards. I am not quite sure I know what the requirements should be; whether these should be set as a minimum to which any reasonably advanced country should adhere or whether these are set as guide lines. If you say it is the minimum, then looking to the USA you can say it is totally insufficient for this 'advanced country'. The guide lines can be interpreted differently or do we set these guide lines on the very maximum? – in which case we in the WHO are bound on the acceptance of vaccines to turn down vaccines which do not meet these standards. Moreover a relevant question when making recommendations is: what should be the international recommended standard? I think we should recognize what we are talking about because I cannot see in this case that the USA does not require its vaccine to come up to the standard recommended at the present time for use by WHO.

CHAIRMAN Since we have some of the experts for WHO among us we should ask them to comment on your remark.

BENENSON (USA) The question of potency of the US Reference Vaccine would seem to really refer to the potency of the WHO Reference vaccine.

COHEN (The Netherlands) If a vaccine does not meet WHO requirements this means that it is not acceptable for WHO vaccination campaigns. Neither can the producer claim the name 'Vaccinum Variolae'. It is a matter of the law applied in the particular country whether the vaccine can be used in this country.

EDSALL (UK) Although Dr Cohen has stated the legal situation with regard to national versus international requirements, perhaps it is worth while to note that, as all legal positions have a practical basis, the legal situation here is consistent with the fact that the characteristics of a vaccine made for domestic use may quite logically be different from those of a vaccine designed to meet the potentially adverse conditions of international shipment and use.

HEKKER (The Netherlands) In titrating vaccinia virus – and not only this virus but also, for instance, rubella virus – we repeatedly found a more or less sudden decrease in sensitivity of monolayers of RK 13 cells after a relatively great number of passages (let us say 50). So that we have to go back to the liquid N_2 and start all over again with fresh cultures.

NETTER (France) We did not check the sensitivity during many passages but the history of the cells is known. They were passed in our laboratory 20 times.

PERKINS (UK) The phenomenon of sudden loss of sensitivity of a cell line to a virus is a common finding with several cell lines and many viruses. It happens, for example, with the BSC 1 cell line used for titrating poliomyelitis virus. It is important therefore to have a number of ampoules of the cell line held in liquid nitrogen as a cell bank and to return to a fresh ampoule after 10–15 passages. It is important also to have a reference preparation of known virus titre available for checking the sensitivity of the cell cultures.

BERNSTEIN (USA) We have observed that maximum adsorption of vaccinia virus on to primary monkey kidney tissue cells occurs between 5 and 8 h. Following 3 h adsorption the titre is 0·3–0·2 ^{10}log lower. On what basis did you select a 3 h adsorption period for the inoculation?

NETTER (France) Many others use an adsorption period of 1 h, another discrepancy in the pH of the medium which varies from 7·4 to 8. This is a question of standardization. Could the chairman ask the participants who is titrating tissue culture and has a general opinion?

CHAIRMAN Dr Netter puts this question in front of us. Do we all share the opinion that WHO or the experts of WHO should take notice of these differences or do we consider the situation as not yet clear enough. What is the opinion of those who work with tissue culture and other titration methods?

TAGAYA (Japan) As far as the smallpox vaccine now in use, primary chick embryo cell monolayers are very good for plaque titration with minimal variations in susceptibility. With regard to TC vaccine, we should not discuss this on the same level, because by serial passages through cultured cells, though less than five passages, there may take place selection of virus population which may lead to discrepancies between the plaque titres obtained and infectivity in man.

MURRAY (UK) At the expense of being repetitive I suggest that the provision of a suitable reference preparation would solve many of the difficulties attending the potency assay of tissue culture smallpox vaccine.

COHEN (The Netherlands) WHO requirements for potency have been established on the basis of evidence that a correlation existed between the take-rate in man and pock counting in CAM. This may be especially true for vaccines prepared on calf skin. Such a relationship should be established also in the case of any new tissue culture vaccine before suitable reference preparation and requirements for potency could be established by WHO.

CHAIRMAN What Dr Netter proposed finally was that the reference preparation should be a standard preparation alotted with a number of units. Do we have anything of that kind for any live vaccine?

OUTSCHOORN (WHO) No.

NETTER (France) Who is actually using tissue culture methods for titration of his own smallpox vaccine as routine? In my laboratory it was only for experimental use; we are routinely using the CAM method.

FULLER (USA) We are checking the US reference vaccine in tissue culture. The titre is $^{10}\log 8{\cdot}1$ per ml when we use 0·1 ml inoculum.

CHAIRMAN We titrate on primary monkey kidney cells because we had a similar experience to Dr Espmark in that the titration on primary monkey kidney cells gives results with narrow confidence limits.

VALLE (Finland) The plaque assay methods used in different laboratories vary a lot. It is known that the sensitivity of a plaque assay system can be increased significantly by optimizing the conditions of the assay method. However, in the control of a vaccine the reproducibility of the assay system is the most important feature of the method and a reference preparation should be included in each assay. When a tissue culture assay system is accepted for the control of smallpox vaccine it is to be expected that variation in the results will be caused by the fact that various virus strains are used in different countries.

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TITRAGE COMPARATIF DU VACCIN ANTIVARIOLIQUE SUR DIFFERENTS SYSTEMES CELLULAIRES ET SUR MEMBRANE CHORIO-ALLANTOIDE D'EMBRYONS DE POULET

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Pour S. E. Luria, l'unité infectieuse est la plus petite quantité de virus qui peut produire une manifestation donnée dans un système sensible, et le titre, est le nombre d'unités infectieuses par unité de volume. Les diverses méthodes de titrage utilisées mettent en jeu l'action infectieuse, l'action léthale, l'effet cytopathogène, l'effet hémagglutinant ou la neutralisation d'une de ces propriétés par des sérums anti-viraux de titre connu. Donc, les résultats des titrages dépendent de la quantité de virus actif, mais aussi des conditions expérimentales et en premier lieu, de la sensibilité du système indicateur vis-à-vis du virus.

Au moment où ce travail a été effectué, l'Organisation Mondiale de la Santé reconnaissait et recommandait (7) une seule méthode pour le titrage du vaccin antivariolique: la numération des pustules obtenues avec des dilutions croissantes d'une suspension vaccinale sur la membrane chorio-allantoïde d'embryons de poulet incubés pendant environ 12 jours. Si les autres méthodes n'étaient pas formellement rejetées, il était dit que leur acceptation impliquait l'acquisition 'd'une plus grande expérience de ces épreuves et des rapports entre leurs résultats et ce que l'on obtient sur les membranes d'embryons de poulet'.

Confrontés depuis plusieurs années avec les problèmes de fabrication et de titrage de ce vaccin, nous avons voulu, tout en restant dans un domaine pratique accessible à tout laboratoire, comparer les résultats obtenus sur des systèmes cellulaires faciles à se procurer ou à entretenir et la membrane chorio-allantoïde.

MATERIEL ET METHODE

Souches cellulaires Notre expérimentation a débuté avec 7 souches cellulaires différentes: cellules de lignée continue humaines (Hela, KB, Hep 2) animales (rein de veau) et cellules de première explantation (embryons de souris, de poulet, cellules de rein de porc). Rapidement, nous avons du ne conserver que les lignées humaines continues par suite du manque de sensibilité des autres systèmes et à cause des difficultés rencontrées pour nous approvisionner, à la demande, en tubes de culture.

Œufs Nous avons utilisé des œufs de poule-race Rhode Island Red, Plymouth rock-embryonnés depuis 12 jours. Mais il est difficile, en pays tropical, d'obtenir des œufs sains non contaminés par pullorose ou viroses aviaires provoquant une mortalité anormale des embryons.

Vaccins Les 14 séries de vaccin expérimenté sont de 3 types: (1) 8 lots de vaccin lyophilisé, préparé par l'Institut sur culture de fibroblastes d'embryons de poulet (lot VEP 1/69 à 8/69) reconstitué au moment du titrage par remise en suspension dans du liquide Hanks; (2) 3 lots de vaccin liquide glycériné préparé sur génisse; (3) 3 lots d'un vaccin de référence mis à notre disposition par le Serum Staten Institut de Copenhague (vaccin lyophilisé, préparé sur mouton).

TECHNIQUES Les différents lots de vaccin dilué de 10–1 à 10–8 dans du liquide Hanks, sont inoculés aux dilutions de 10–5 à 10–8 (dilutions couvrant généralement l'échelle de 0 à 100/100 de survie):

– d'une part, à des séries d'œufs embryonnés supérieures à 10 (généralement de 12 à 15), pour éliminer les œufs morts en cours de titrage pour des raisons autres que l'infection vaccinale. On inocule 0,2 ml de suspension. Après fermeture de l'orifice d'inoculation au scotch, les œufs sont remis à l'étuve à 37 °C pendant 3 jours, temps au bout duquel on effectue la lecture.

– d'autre part, à des séries de 10 tubes de culture des 3 souches cellulaires utilisées à raison de 0,1 ml par tube pour chacune des 4 dilutions retenues. L'absorption se poursuit pendant une heure à l'étuve à 37 °C, puis, après avoir ajouté le milieu de survie: Hanks caseine sérum de veau à 5 %, tous les tubes sont mis à incuber à l'étuve à 37 °C en tambour roulant. Le contrôle est effectué quotidiennement et la culture poursuivie pendant trois semaines, avec un changement de milieu tous les 5 jours pour les cellules Hela et un changement tous les 2 jours pour les cellules KB et Hep. 2.

Calculs Seuls les lésions indiscutables de la membrane chorio-allantoïde ont été retenues: pustules plus ou moins isolées, quelquefois hémorragiques, opacités blanches, circulaires, présentant habituellement une dépression en forme de cratère.

Sur cultures cellulaires, les lésions sont plus ou moins rapidement décelables en fonction de la dilution: grappes de cellules réfringentes évoluant vers une plage nécrotique à bordure granuleuse, brune; apparition de plages satellites; destruction finale de tout le tapis cellulaire.

Tous les calculs (DCP 50 et DL50) ont ensuite été effectués selon la méthode des totaux cumulatifs de L. J. Reed et H. Muench, et les résultats rapportés au millilitre de suspension vaccinale.

RESULTATS

La méthode d'analyse utilisée est applicable aux cas de 'deux facteurs contrôlés combinés factoriellement' ou à 'un plan d'expérience à deux facteurs sans répétition'.

Elle a montré que pour le seuil de 5 %, il n'y a pas de différence significative entre les diverses méthodes de titrage utilisées, alors que cette différence existe entre les différents lots de vaccin étudiés.

De même, elle a montré que les différences entre les moyennes des diverses méthodes pourraient être considérées comme significatives au seuil 0,05, si elles dépassaient 0,230. Or, les moyennes obtenues avec les 4 systèmes de titrage utilisés sont: Hela 7,83; KB 7,82; Hep. 2 7,61; œufs 7,68.

Table I. *Comparaison des résultats obtenus, pour différents lots de vaccin, avec différents systèmes de titrage*

Lots de vaccin		Echantillons Système de titrage			MCA œufs
		Hela	KB	Hep. 2	
VEP 1	1	7,50	7,20	6,40	7,86
VEP 2	2	8,00	7,72	7,78	7,70
VEP 3	3	7,83	7,82	7,57	7,23
VEP 4	4	7,62	7,82	7,90	9,00
VEP 5	5	8,28	8,12	7,91	8,40
VEP 6	6	8,37	7,81	7,50	7,40
VEP 7	7	8,17	8,18	7,61	7,50
VEP 8	8	7,83	8,23	7,80	7,34
Génisse 1	9	8,33	8,62	8,27	8,02
Génisse 2	10	8,28	8,60	8,18	7,86
Génisse 3	11	7,71	8,00	8,00	7,72
OMS. 1	12	7,62	7,55	7,50	7,93
OMS. 2	13	6,83	6,62	6,83	7,36
OMS. 3	14	7,37	7,29	7,42	7,25
Moyenne		7,83	7,82	7,61	7,68

DISCUSSION

L'effet cytopathogène est dû à la pénétration du virus dans la cellule dans laquelle il se reproduit. Sa croissance détruit la cellule et les virions libérés infectent les cellules voisines de proche en proche. La destruction des cellules infectées au moment de l'inoculation donne une plage primaire (par suite de l'action de 10 particules infectieuses environ(**8**)), les virions secondairement libérés dans le milieu provoquent la formation de plages secondaires. L'apparition de ces plages secondaires gène l'interprétation des méthodes de titrage utilisant la numération des plages. Kirn(**2–4**) insiste sur le fait qu'il faut effectuer la lecture avant leur apparition, ce qui limite le temps d'observation. L'apparition de ces plages est de plus soumise à l'action de différents facteurs non spécifiques: nature du milieu, concentration du sérum, conditions générales de culture, volume d'inoculum, température, agrégation plus ou moins importante des particules, temps d'adsorption(**1–4, 10**).

L'établissement d'une DCP 50 n'a pas les mêmes inconvénients puisqu'elle cherche seulement à obtenir, avec plusieurs dilutions consécutives, un effet cytopathique allant d'une atteinte généralisée de tous les tubes à un effet nul. L'observation peut alors être poursuivie autant que le permet la résistance des cellules en culture, tout effet primarie ou secondaire dû au virus intervenant de la même manière dans le mode d'évaluation des lésions.

La valeur des méthodes de titrage en cultures cellulaires est admise par de nombreux auteurs et si les chiffres obtenus sont généralement inférieurs au

'pock count' sur MCA, ils sont cependant voisins. Par numération des plages sur cellules d'embryon de poulet, Porterfield et Allison obtiennent des titres du même ordre de grandeur que ceux obtenus avec le 'pock count' et trouvent même la méthode plus reproductible. Postlethwaite(**9**) admet que le rapport entre les titres obtenus par les deux méthodes est inférieur à 2.

Le titrage recommandé sur MCA d'embryons de poulet n'est d'ailleurs pas exempt d'inconvénients. D'après R. Camain *et al.*, le seul reproche que l'on puisse faire à la MCA en temps que matériel d'analyse, est son extrême sensibilité. Ces auteurs ont obtenu à partir d'agents d'agression très divers (désinfection à l'alcool iodé, ouverture de la coquille, dépression de la MCA, variation de température et d'humidité pendant l'incubation, variation de pH ou de pression osmotique de l'inoculum), des images pouvant prêter à confusion avec celles provoquées par des inoculations de suspension virale. Ils soulignent d'autre part, l'inconstance des lésions obtenues sur des œufs traités de façon identique par un même expérimentateur. Lors du travail effectué en vue d'établir une préparation vaccinale internationale de référence, Krag *et al.*(**5**) experiment certains résultats en 'value based on reading including less well defined pocks', ce qui illustre bien les difficultés qu'il y a parfois à interpréter les résultats obtenus sur MCA, par 'pock count' comme par DL 50, cette dernière étant cependant plus facile à établir.

Ceci est d'ailleurs une des raisons qui nous ont amené à choisir, non le 'pock count', mais la détermination de la DL 50, qui, de l'avis de la plupart des auteurs, est un test plus sévère. Ainsi, Krag *et al.* (**5**) donne pour la suspension de référence les titres suivants: $10^{8,4}$ par ml en 'pock count', $10^{7,9}$ par ml en DL 50, sur œufs.

De plus DCP 50 et DL 50 sont des méthodes plus facilement comparables que DCP 50 et numération des pustules (**6**). L'une et l'autre évaluent la proportion d'unités réactives au sein d'une population donnée. Au contraire, la méthode de numération des pustules évalue le nombre d'unités virales formatrices de lésions caractéristiques.

CONCLUSION

L'utilisation des quatre systèmes en parallèle: cultures cellulaires Hela, KB, Hep. 2 et DL 50, sur œufs ne nous a pas permis de mettre en évidence une différence significative entre les titres obtenus. C'est pourquoi, il nous paraît logique de proposer, en dehors de la méthode recommandée sur MCA, la méthode de titrage en cultures cellulaires.

La DCP 50 sera établie sur 10 tubes par dilution. L'échelle des dilutions doit donner des lésions allant d'une destruction totale à l'absence d'effet cytopathique. L'observation sera poursuivie pendant deux semaines, temps après lequel on n'observe plus aucun effet aux dilutions utilisées; sur près de 2000 tubes observés, seuls 2 ont été positifs après ce délai.

Cette méthode est à la portée de tout laboratoire entretenant des cellules de souche et de réalisation pratique facile. Les laboratoires fabriquant du vaccin

antivariolique pourraient aussi disposer, non seulement de vaccin de référence, mais également de souches cellulaires contrôlées, définies et de sensibilité connue.

RESUME

Les auteurs, après avoir comparé les résultats des titrages obtenus par les différentes méthodes, critiquent l'utilisation de la MCA et justifient leur choix d'une technique faisant intervenir la DCP 50 sur culture de cellules. Cette méthode, à la portée de tout laboratoire entretenant des cellules de souche, est à la fois pratique, fidèle et sûre.

Nous remercions MM. Gillibert, Docteur Vétérinaire à l'IEMVPT et Gendreau, statisticien ORSTOM, de leurs précieux conseils.

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ETUDE DE LA SENSIBILITE DE QUELQUES LIGNEES CELLULAIRES POUR TITRER LE VACCIN ANTIVARIOLIQUE

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Le virus vaccinal est cultivable sur un grand nombre de cellules fibroblastiques ou épithéliales provenant d'animaux les plus variés à sang froid ou à sang chaud et chaque auteur, selon le but poursuivi, adopte un système cellulaire particulier.

En dépit des travaux déjà anciens (**1, 2, 3, 6, 7**) montrant le caractère reproductible des titrages et la sensibilité de certains systèmes cellulaires au virus vaccinal, le test de référence de l'Organisation Mondiale de la Santé reste le titrage sur membranes chorio-allantoïdiennes (MCA) d'œufs embryonnés. Notre but est de reprendre un travail antérieur sur cellules rénales simiennes (**5**) et d'étudier si certaines lignées cellulaires courantes dans les laboratoires pourraient être aussi utilisées avec la même commodité, la même précision et la même sensibilité.

MATERIEL

Vaccins antivarioliques

Il s'agit des vaccins antivarioliques frais ou lyophilisés (souche Chambon) que nous recevons régulièrement pour contrôle. Dans le cas des vaccins lyophilisés, la reconstitution est faite avec la solution glycérinée tamponnée à pH 7,3–7,5 délivrée par le fabricant.

Les cellules

HeLa Cette lignée de cellules épithéliales humaines nous a été remise par le Dr Chany, en 1963; elle est entretenue depuis dans notre laboratoire. Des vérifications ont montré qu'elle était contaminée par *Mycoplasma orale*. Le milieu de croissance est à base de Eagle MEM enrichi de 10 % de sérum inactivé de veau, additionné de bicarbonate et d'antibiotiques (Pénicilline-Streptomycine). Les tubes à fond plat (Leighton-Barski) sont ensemencés avec 2×10^5 cellules sous le volume de 2 ml; à cette concentration, le tapis cellulaire est pratiquement confluent en 3–4 jours.

RK 13 Cette lignée de cellules épithéliales rénales de lapin provient du Laboratoire du Dr Stoker de Birmingham; elle nous est parvenue en 1965 par l'intermédiaire du Prof. Sohier de Lyon. Des vérifications ont montré qu'elle était indemne de mycoplasmes. Pour la culture, on utilise le même milieu de croissance que pour les cellules HeLa. Les tubes à fond plat sont ensemencés avec 3×10^5 cellules sous le volume de 2 ml; à cette concentration, le tapis cellulaire est confluent en 3–4 jours.

MA 104 Cette lignée de cellules épithéliales rénales de singe nous est parvenue en 1969 par l'intermédiaire du Prof. Sohier de Lyon. Nos contrôles ont montré que les cellules

étaient contaminées par des mycoplasmes. Pour la culture, on utilise le même milieu de croissance à base de Eagle MEM et de 10 % de sérum de veau précédemment décrit. Les tubes à fond plat reçoivent chacun 2×10^5 cellules sous le volume de 2 ml; la confluence est obtenue en 3–4 jours.

Les œufs embryonnés

Ils sont de race Leghorn et sont incubés dans notre propre laboratoire pour être inoculés aux 11e–12e jour.

METHODES

Dilution des vaccins

Les vaccins, frais ou lyophilisés reconstitués, sont dilués de 10 en 10 avec une solution de Earle sans chlorure de calcium, ni sulfate de magnésium, additionnée de 0,35 % d'Albumine bovine 'Armour' et dont le pH est ajusté à 7,4–7,6. On utilise pour le titrage deux dilutions successives de vaccin et les mêmes suspensions virales sont inoculées le même jour aux cultures cellulaires et aux MCA d'œufs embryonnés; nous éliminons ainsi le facteur de variation dû au pipetage ou à la nature du diluant.

Titrage sur cultures cellulaires

On utilise 5 à 10 tubes par dilution et après élimination du milieu de croissance on dépose 0,4 ml de suspension virale appropriée dans chaque tube; ce volume nous a paru nécessaire pour obtenir une bonne dispersion des plages. L'adsorption du virus dure 3 h à 37 °C, puis on complète chaque tube avec 1,5 ml de milieu de Eagle contenant 5 % de sérum et dont le pH est compris entre 7,2–7,5. Cette concentration de sérum est nécessaire pour obtenir des plages d'une taille suffisante pour être dénombrées à l'œil nu; mais, comme la croissance cellulaire n'est pas interrompue, le milieu d'entretien doit être plus fortement tamponné que le milieu de croissance.

Après 2 jours d'incubation des tubes à 37 °C en position inclinée, on remplace le milieu d'entretien par une solution de rouge-neutre à 1 % pendant 30 min à 37 °C, puis on rejette l'excès de colorant; il reste alors à dénombrer les plages dans chaque tube, à l'œil nu ou à l'aide d'une loupe faiblement grossissante, et à calculer ensuite le titre du vaccin par ml en tenant compte de sa dilution et du volume.

Titrage sur œufs embryonnés de poule

On utilise 5 à 10 œufs embryonnés par dilution et après effondrement de la MCA suivi d'un temps de latence d'environ une demi-heure, on dépose à la surface de chaque membrane 0,1 ml de la suspension virale; on obture l'orifice de la coquille avec de l'adhésif, puis on remet les œufs en position stationnaire pendant 2 jours à 36 °C. Le prélèvement de la MCA est suivi du dénombrement des pustules (PFU) et on calcule ensuite le titre du vaccin par ml en tenant compte de sa dilution et du volume inoculé.

RESULTATS

Nous donnons dans les Tableaux I–III les résultats obtenus sur des vaccins frais et lyophilisés titrés le même jour sur MCA d'œufs embryonnés et sur cellules.

Un premier coup d'œil sur les Tableaux I à III fait ressortir les observations suivantes.

Tableau I: le titrage du vaccin sur MCA est 5 fois plus sensible que sur cellules Hela ($10^{0,7}$).

Tableau II: le titrage du vaccin frais sur MCA est 3,6 fois plus sensible que sur cellules RK 13 ($10^{0,55}$), ce qui représente déjà une amélioration par rapport au

Tableau I. *Titrages comparés sur MCA d'œufs embryonnés et sur cellules HeLa*

	Vaccins antivarioliques			
	Frais		Lyophilisés	
	MCA	HeLa	MCA	HeLa
Nombre de vaccins (n)	13	13	8	8
Titre moyen par ml	$10^{8,04}$	$10^{7,34}$	$10^{8,17}$	$10^{7,85}$
Variance (S^2)	$10^{15,5}$	$10^{14,7}$	$10^{14,36}$	$10^{14,8}$
Ecart-type (S)	$10^{7,75}$	$10^{7,34}$	$10^{7,17}$	$10^{7,4}$
Intervalle de confiance pour $P = 0,05$ ($\pm t.S/\sqrt{n}$)	$\pm 0,34$	$\pm 0,13$	$\pm 0,12$	$\pm 0,21$

Tableau II. *Titrages comparés sur MCA d'œufs embryonnés et sur cellules RK* 13

	Vaccins antivarioliques			
	Frais		Lyophilisés	
	MCA	RK 13	MCA	RK 13
Nombre de vaccins (n)	12	12	9	9
Titre moyen par ml	$10^{8,15}$	$10^{7,6}$	$10^{8,37}$	$10^{7,86}$
Variance (S^2)	$10^{15,38}$	$10^{15,63}$	$10^{16,66}$	$10^{15,46}$
Ecart-type (S)	$10^{7,70}$	$10^{7,81}$	$10^{8,33}$	$10^{7,73}$
Intervalle de confiance pour $P = 0,05$ ($\pm t.S/\sqrt{n}$)	$\pm 0,31$	$\pm 0,41$	$\pm 1,64$	$+0,42$

Tableau III. *Titrages comparés sur MCA d'œufs embryonnés et sur cellules MA* 104

	Vaccins antivarioliques			
	Frais		Lyophilisés	
	MCA	MA 104	MCA	MA 104
Nombre de vaccins (n)	8	8	24	24
Titre moyen par ml	$10^{8,04}$	$10^{7,74}$	$10^{8,45}$	$10^{8,09}$
Variance (S^2)	$10^{15,8}$	$10^{15,2}$	$10^{16,66}$	$10^{15,78}$
Ecart-type (S)	$10^{7,9}$	$10^{7,6}$	$10^{8,3}$	$10^{7,9}$
Intervalle de confiance pour $P = 0,05$ ($\pm t.S/\sqrt{n}$)	$\pm 0,65$	$\pm 0,33$	$\pm 0,85$	$\pm 0,33$

titrage sur cellules HeLa, tandis que le titrage du vaccin lyophilisé sur MCA est 3,20 fois plus sensible que celui sur cellules RK 13 ($10^{0,5}$). Le résultat est assez voisin de celui déjà obtenu pour les mêmes cellules avec le vaccin frais.

Tableau III: Pour le vaccin frais, le rapport entre les deux modes de titrage n'est que de 2 ($10^{0,3}$), toujours en faveur du titrage sur œufs. Pour le vaccin lyophilisé, le rapport entre les deux modes de titrage est de 2,3 ($10^{0,36}$), chiffre voisin de celui antérieurement trouvé pour les vaccins antivarioliques frais titrés sur les mêmes cellules.

DISCUSSION

Des trois systèmes étudiés, c'est sur cellules RK 13 que les plages sont les plus visibles à l'œil nu, la lecture à la loupe ou au microscope n'améliorant pas les résultats. Avec les cellules MA 104, les plages sont un peu plus petites et leur dénombrement à l'œil nu nécessite un certain entraînement.

Pour un même type de cellules, nous n'avons pas constaté de différence de sensibilité des titrages de vaccins frais ou lyophilisés.

La sensibilité des cellules Hela par rapport à celle des MCA d'œufs embryonnés est très variable d'un laboratoire à l'autre: identique pour Cutchin & Warren(**3**), 1 à 2 fois plus faible pour Nishmi & Keller(**6**), 3 à 5 fois plus faible dans nos propres expériences et 7 fois dans celle de Valle(**9**). Dans ce dernier cas, la faible durée du temps d'adsorption, qui n'est que d'une heure, et le fait de titrer les plages dans des tubes ronds, sont sans doute en cause. Dans notre propre cas, nous pensons que le pH du milieu d'entretien aurait dû être de 7,8–8(**6**) au lieu de 7,2–7,5, mais d'autres facteurs peuvent également intervenir, comme le fait d'employer une lignée cellulaire HeLa différente.

La comparaison des titrages sur cellules RK 13 et sur MCA d'œufs embryonnés nous a donné un rapport de 3,2 à 3,6, alors que pour d'autres(**9**), il est de 8,1. A noter que sur cellules rénales primaires de lapin, Cutchin & Warren(**3**) obtiennent là encore la même sensibilité que sur MCA.

Il semble y avoir une meilleure concordance quant à la sensibilité des cellules simiennes: avec la lignée MA 104, la sensibilité n'est réduite dans notre laboratoire que de 2 à 2,3 ($10^{0,3}$ à $10^{0,36}$). Sur cellules GMK et SVEA, Valle(**9**) observe également une réduction du titre de 1,5 à 2,4 par rapport à celui déterminé sur MCA. Cutchin & Warren(**3**) d'une part, et Espmark(**4**) d'autre part trouvent aux cellules rénales primaires une meilleure sensibilité que les MCA d'œufs embryonnés.

Sur d'autres cultures primaires de fibroblastes, provenant d'embryons de poulet cette fois, Postlethwaite(**7**) observe des résultats identiques à ceux obtenus sur MCA, alors que pour d'autres(**2, 8, 9**) la sensibilité est un peu moins bonne si on tient compte du fait que les résultats sur MCA sont donnés en PFU et ceux sur fibroblastes en TCD 50. Pendant longtemps, on a reproché aux titrages sur MCA d'être variables d'un laboratoire à l'autre; une meilleure standardisation de la technique permet maintenant d'avoir d'assez bons résultats; c'est ainsi que

la moyenne des résultats de 10 titrages de vaccins lyophilisés pris au hasard n'a accusé pour l'un de nos fabricants qu'une différence de $10^{0,08}$ par rapport à nos propres résultats et pour l'autre fabricant une différence de $10^{0,1}$. Le travail est sans doute plus complexe pour les cultures cellulaires, car il existe de nombreuses variétés de cellules, de milieux et de techniques, différentes d'un laboratoire à l'autre.

Si les épreuves sur animaux, sur œufs embryonnés et sur cultures cellulaires sont indispensables pour caractériser une souche ou un lot de semence, en revanche, les titrages de routine pourraient très bien être pratiqués en culture cellulaire.

Comme le titre de 10^8/ml repris par l'OMS est basé sur des titrage sur MCA d'œufs embryonnés, il est possible que certains vaccins ne satisfont pas aux normes s'ils sont titrés en cultures cellulaires, puisque celles-ci se sont révélées moins sensibles dans un certain nombre de laboratoires, l'adjonction, au cours du même titrage, d'un vaccin de référence titré sur MCA permettra dans ce cas d'effectuer les corrections nécessaires, et nous ne doutons pas que sous l'égide de l'OMS et de la Societé de Standardisation, il soit possible d'arriver rapidement à un résultat.

CONCLUSIONS

La numération des plages en milieu liquide et après 48 h d'incubation est facile à réaliser. Des trois lignées étudiées, celle de singe présente la meilleure sensibilité et semble donc préférable pour un travail de routine.

La comparaison de nos résultats avec ceux d'autres auteurs montre un certain nombre de variations et assez souvent une moindre sensibilité des cellules par rapport aux titrages effectués sur MCA d'œufs embryonnés. L'emploi d'un vaccin de référence présentant le titre requis sur MCA permet, dans ce cas, de savoir si le vaccin testé répond aux normes de 10^8/ml fixées par l'OMS.

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STABILIZATION OF LIQUID SMALLPOX VACCINE

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The heat-instability of viruses has prompted a search for suitable stabilizers, both for routine storage for laboratory studies, and also for the development of live vaccines suitable for use in tropical climates lacking refrigeration facilities. This need is particularly acute in the case of smallpox vaccine. Although freeze-drying has made possible the maintenance and use of smallpox vaccine on a large scale in warm climates, a stable liquid vaccine, if available, would be more easily handled and more economical to use. The studies to be reported here were undertaken primarily with a view to the development of a liquid smallpox vaccine with adequate heat-stability to retain its effectiveness for reasonable periods, at high ambient temperatures without refrigeration.

Proteins and protein derivatives have long been recognized as having virus stabilizing properties(**6**, **7**, **8**) in a review of the literature on the preservation of vaccinia virus, observed that peptone was the most satisfactory of the several substances then tested. In a more recent study Amies(**1**) compared the protective efficacy of peptone, gelatin and bovine serum albumin on the heat-inactivation of vaccinia virus and reported that peptone was superior. Amies found even greater protection with polyvinyl pyrrolidone (PVP), but in contrast to peptone, PVP was rather ineffective in the presence of phenol. For practical purposes of vaccine preparation, Amies therefore recommended a saline solution containing both peptone (1%) and PVP (12·5%) with phenol as the bacteriostatic agent.

Inorganic ion concentrations have also been known to affect the stability of viruses(**8**, **12**) and recent studies have revealed an interesting relationship in this respect. Thus RNA viruses are protected by high concentrations of divalent cations (1 M) while DNA viruses including vaccinia are inactivated by such concentrations of divalent cations but are protected by monovalent cations such as 2 M-NaCl(**16**).

Cysteine has been reported to protect vaccinia virus against heat(**13**) but these results have not been confirmed(**5**, **11**). Amos'(**2**) studies with Herpes simplex virus suggest that cysteine might protect some viruses indirectly through inactivation of tissue phosphatase enzymes. Various other substances, including sugars, milk solids, fats and emulsifiers, have been tested with vaccinia virus but differences of opinion exist as to their protective efficacy(**5**). Saccharose and glutamic acid, singly or in combination, have been found to be effective stabilizers

for rickettsiae(3). Glycerol has long been used both as a stabilizer and a bacteriostatic agent in the preparation of smallpox vaccine. Some have found it to be comparable to broth, etc., at least when tested at low temperatures(9). others have found it to be inhibitory to the vaccinia virus(1, 15, 16). It has been suggested that the inhibitory action of glycerol might be due to the development of acidity during storage(5).

MATERIALS AND METHODS

All substances were tested in 1 % concentration unless otherwise stated. They were dissolved in physiological saline buffered with 0·01 M phosphate to pH 7–7·2 or in Hank's buffered salt solution (BSS) with phenol red (without glucose), buffered with 0·01 M Tris to pH 7·4.

The vaccinia virus strain routinely used in these laboratories for the preparation of smallpox vaccine was employed in this study. The virus was first purified from the crude calf pulp by fractional centrifugation according to the method of Amies (1). It was kept frozen in distilled water buffered to pH 7–7·2 with 0·004 M phosphate citrate buffer. Prior to use it was diluted 1:100 in the appropriate medium. Tubes containing aliquots of the preparations under study were immersed in a water bath at either 37 or 47 °C for the periods of time indicated, after which the contents were titrated for viable vaccinia virus.

The virus was assayed by the plaque technique in tissue cultures of chick embryos. The method of Porterfield & Allison (14) was employed. The plating medium was tris-buffered medium 199 with 1 % horse serum, shown to be non-inhibitory to the vaccinia virus. The test was carried out in disposable Petri dishes measuring 60 × 15 mm. Initial experiments were carried out to determine the optimum conditions for virus adsorption. A virus inoculum volume of 0·5 ml in tris-buffered medium 199 with 1 % BSA and an adsorption period of 4 h at 37 °C was adopted. The overlayed medium was tris-buffered 199 with 15 % horse serum and 1 % agar. Plaques were developed with neutral red after 4 days incubation.

RESULTS

Representatives of various groups of substances that have been reported to protect vaccinia or other viruses were tested in the hope that this property might be shared by other members of the group. A few substances were tested both at 37 °C and 47 °C. As was to be expected, a significant (though incomplete) parallelism was found. Most experiments were therefore carried out at 47 °C because of the rapidity with which the desired information could be obtained. At 47 °C the titres of vaccinia virus suspended in saline or Hank's BSS falls from 5×10^6 pfu/ml to less than 1 pfu/ml within 24 h. Heating at 47 °C for 24 h was therefore used as a screening test. This temperature was also preferred because it was felt that the need for stabilization of vaccinia virus was probably more critical at very high ambient temperatures. Substances that failed to permit survival of the virus in detectable quantities were considered 'unprotective' and were usually not investigated further.

Tests carried out at 37 °C (Fig. 1) revealed that cysteine and PVP were inhibitory. Dextran, which gave slight protection at 47 °C, was found to be slightly inhibitory at 37 °C, while polyvinyl alcohol (PVA) – another one of the substances that was slightly protective at 47 °C – did not markedly affect the rate of inactivation of the virus at 37 °C.

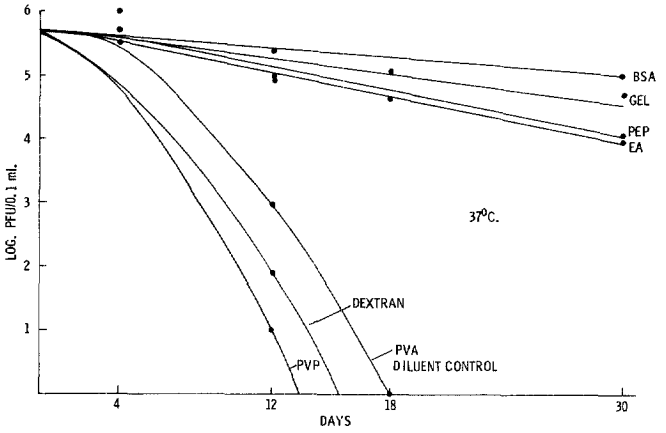

Fig. 1. Inactivation of vaccinia virus in various candidate stabilizers at 37 °C. BSA, Bovine serum albumin; HSA, human serum albumin; GEL, gelatin; PEP, peptone; EA, egg albumin; PVA, polyvinyl alcohol; PVP, polyvinyl pyrrolidone; LYS, lysine; GLY, glycine; MACS, macromolecules.

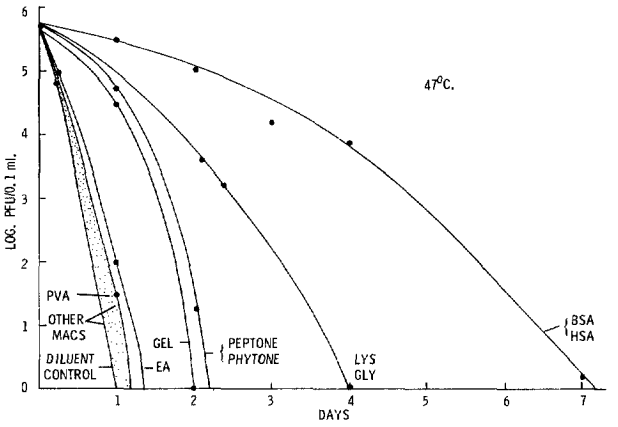

Fig. 2. Inactivation of vaccinia virus in various candidate stabilizers at 47 °C.

All the proteinaceous substances tried were found to be protective (Figs. 1, 2). All the amino acids tried likewise proved to be effective stabilizers. The most impressive results by far were obtained with the serum albumins. This was particularly evident at 47 °C and was observable at 37 °C. Of the amino acids, glycine and lysine were the most effective.

The protective effect of serum albumins survived heat-denaturation to the point of coagulation, but HSA which had been sterilized by heating at 60 °C for 10 h in the presence of caprylate and *n*-acetyl tryptophanate(4) was considerably less effective. The protective action of the serum albumins was unaffected by the simultaneous presence of the amino acids glycine, lysine and

glutamic acid but was considerably reduced in the presence of 50% glycerol. Slightly better protection could be detected with BSA dissolved in 2 M sodium lactate.

DISCUSSION

The results presented here, while in general confirming the marked stabilizing action of proteins on viruses, differ from other published reports in a few specific areas. PVP in 1% concentration (and even more so at 12·5% concentration) was found to be inhibitory in contrast to the results reported by Amies(**1**). No satisfactory explanation for this discrepancy can be given at this time. However, one may assume that the spectrum of aggregation in the preparations used by Amies and by us may have been significantly different.

The results presented here differ also from those reported by Amies(**1**) in the superior stabilization achieved in our studies with the serum albumins as compared with peptone. Amies' tests were carried out only at temperatures at or *below* 37 °C and, as our results indicate, the superiority of the serum albumins over peptone is not so well marked at this temperature. On the other hand, Amies' results with peptone are remarkably similar to our own.

The stabilization of the vaccine may have been facilitated by the use of diluted material. Preliminary studies indicated that preparations of comparable potential stability could be obtained by partial purification of full-strength vaccine in a high-speed centrifuge, as recommended by Collier(**5**) for freeze-dried vaccine. Other methods, such as gel-filtration(**10**), might serve as well or better. The exact preparation of a human serum albumin solution of optimal stabilizing power and free from the serum hepatitis virus also needs more detailed consideration. Nevertheless, whether the stabilizer of choice is HSA or some other substance or combination, the findings presented here indicate, in principle, that the preparation of a highly heat-stable liquid smallpox vaccine without great expense is entirely feasible.

SUMMARY

Studies on the efficacy of various substances in stabilizing liquid smallpox vaccine indicated that best results were obtained with bovine or human serum albumin.

Bovine vaccine to which was added 1% of either of these substances lost no more than about 0·5 log in virus titre after 3–4 weeks incubation at 37 °C or 48 h at 47 °C.

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FACTORS INFLUENCING THE STABILITY OF FREEZE-DRIED SMALLPOX VACCINE

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The technique of freeze-drying virus suspensions affords the best way of obtaining many viral vaccines in a high state of potency and stability. This is especially true of smallpox vaccine. However, some batches of freeze-dried smallpox vaccine are neither potent nor stable; these preparations subsequently fail the statutory stability tests and are rejected. In this laboratory an investigation was started on some of the factors involved in the stability of freeze-dried smallpox vaccine. Only three factors were investigated:

(i) the residual water after freeze-drying,

(ii) the oxidation of sulphydryl groups, and

(iii) the total nitrogen content of the vaccine.

Other factors may be concerned in the loss of viral stability, but the three selected seemed to be the most obvious point from which to start this investigation. Earlier experiments in this field (**7**) confirmed this view.

Much of the earlier work on residual water and stability of freeze-dried viruses was done by Grieff & Rightsel (**2, 3, 4**). These authors stressed that both overdrying and underdrying could result in the loss of viral stability. The suggestion that oxidation of sulphydryl to disulphide groups is also involved was due mainly to Levitt (**6**). The presence of large amounts of extraneous host protein in many viral vaccines is known to afford some protection against loss of stability after freeze-drying. The total nitrogen therefore was measured in all the experiments.

MATERIALS AND METHODS

The residual water in the freeze-dried vaccine was estimated by the gas chromatographic method of Robinson (**9**). The vaccine sample was extracted with rigorously dried benzene and the water estimated by gas chromatography. A Perkin-Elmer model F. 11 gas chromatograph with a Katherometer detector was used. The carrier gas was high-purity hydrogen at 24 kgf/cm^2 flowing at 80 ml/min. The benzene contained a known amount of *n*-pentane as an internal standard. A calibration curve was obtained by plotting the amount of water against the ratio of the peak height of water over the peak height of *n*-pentane. A simple device (**9**) allowed the sampling of glass-sealed ampoules with no risk of contamination by atmospheric water. The dry weight of the vaccine was found and the water content expressed as weight %.

Table I. *Virus suspension in water (total* N_2 = 13·5 %)

Residual water (wt %)	–SH groups (μmoles/ml)		Virus stability ($\log_{10}$ pfu/ml)	
	−15 °C	4 weeks at 37 °C	−15 °C	4 weeks at 37 °C
2·78	0·175	0·10	7·0	< 3·0
1·60	0·300	0·10	7·2	< 3·0
0·57	0·375	0·10	7·5	< 3·0

Table II. *Virus suspension in* 0·005 M *buffer (total* N_2 = 13·0 %)

Residual water (wt %)	–SH groups (μmoles/ml)		Virus stability ($\log_{10}$ pfu/ml)	
	−15 °C	4 weeks at 37 °C	−15 °C	4 weeks at 37 °C
4·55	0·175	0·10	7·4	< 3·0
1·45	0·300	0·10	7·1	< 3·0
0·61	0·375	0·10	7·4	< 3·0

Sulphydryl groups were determined by the amperometric method of Benesch, Lardy & Benesch (1). The half-cell consisted of a spiral platinum electrode rotating at 80 rev/min.

Total nitrogen was found by the direct estimation of ammonia in total micro-Kjeldahl digests by the method of Jones (5).

Freeze-dried smallpox vaccine containing varying amounts of residual water were obtained by drying for different periods of time in the Edwards 30 P2TS shelf-dryer. The chamber was modified so that each shelf could be sealed independently. No secondary drying cycle was used in any of the experiments. The virus preparation was suspended before drying in water, 0·005 M potassium phosphate buffer at pH 7·2, 5 % peptone in water or in 5 % peptone in 0·005 M buffer. The volume of vaccine in each ampoule was 0·2 ml. Potassium salts were used since it was found that due to differences in solubility between disodium and monosodium salts, a pH of 7·2 was not always maintained.

Vaccinia virus was grown on roller-bottle cultures of chick fibroblasts and titrated on monolayers of the same cell substrate. Titres are expressed as plaque forming units, (pfu/ml). The virus was subsequently purified and concentrated by a partition method (10).

RESULTS

The experimental results are shown in Tables I–VI. The vaccine samples recorded in Tables I–IV were from a single batch of smallpox vaccine, freeze-dried and tested at the same time. A further batch of smallpox vaccine provided the samples used for the experiments recorded in Tables V and VI.

Samples of the vaccine were isolated from the freeze-dryer after 4, 6, 8 and 20 h of the primary drying cycle. Both batches of vaccine contained 0·77 μmoles/ml of –SH groups before freeze-drying. The results in Tables I and II show that the freeze-drying of smallpox vaccine in the absence of peptone resulted in a considerable loss of –SH groups. The results also show the lack of thermal

Table III. *Virus suspension in* 5% *peptone in water (total* N_2 = 32·7%)

Residual water (wt %)	–SH groups (μmoles/ml)		Virus stability $\log_{10}$ pfu/ml	
	−15 °C	4 weeks at 37 °C	−15 °C	4 weeks at 37 °C
2·28	0·560	0·450	7·7	4·8
1·75	0·450	0·450	7·7	7·9
1·18	0·375	0·350	7·9	7·0

Table IV. *Virus suspension in* 5% *peptone in* 0·005 M *buffer (total* N_2 = 32·7%)

Residual water (wt %)	–SH groups (μmoles/ml)		Virus stability ($\log_{10}$ pfu/ml)	
	−15 °C	4 weeks at 37 °C	−15 °C	4 weeks at 37 °C
3·37	0·560	0·745	8·0	3·0
1·86	0·450	0·450	8·0	7·8
1·28	0·400	0·370	7·9	7·6

Table V. *Virus suspension in water (total* N_2 = 14·2%)

Residual water (wt %)	–SH groups (μmoles/ml)		Virus stability ($\log_{10}$ pfu/ml)	
	−15 °C	4 weeks at 37 °C	−15 °C	4 weeks at 37 °C
3·75	0·300	0·175	7·4	< 3·0
2·30	0·320	0·175	7·5	< 3·0
1·50	0·300	0·165	7·6	< 3·0
0·45	0·275	0·150	7·4	< 3·0

stability of this vaccine at 37 °C. The same loss of –SH groups and thermal instability was shown by the second batch of smallpox vaccine when peptone was omitted (Table V). A large reduction of –SH groups occurred during freeze-drying, and a further loss followed after storage of the vaccine for 4 weeks at 37 °C. The presence of 5% peptone prevented the loss of most of the –SH groups during freeze-drying and, after 4 weeks incubation at 37 °C, at least 50% remained. This can be seen in Tables III and IV. The peptone contributed almost no –SH groups, less than 0·10 μmoles/ml. The first samples of vaccine containing peptone (Tables III, IV), isolated from the freeze-dryer after only 4 h drying, were not stable. But after 8 h the vaccine, with a residual water content of about 2.0 wt %, was completely stable after 4 weeks incubation at 37 °C.

Table VI. *Virus suspension in* 5% *peptone in* 0·005 M *buffer* (*total* N_2 = 28·3%)

Residual water (wt %)	–SH groups (μmoles/ml)		Virus stability ($\log_{10}$ pfu/ml)	
	−15 °C	4 weeks at 37 °C	−15 °C	4 weeks at 37 °C
3·55	0·675	0·675	7·8	7·6
1·93	0·675	0·675	8·0	8·0
0·97	0·625	0·600	8·0	8·0
0·85	0·525	0·500	7·6	7·0

DISCUSSION

It is well known that the water in a virus particle in suspension is present at different levels of organization. The 'free water' in which the virus particle is suspended and the 'bound water' which is in much closer association with the protein structure is an obvious simplification but is sufficient for this discussion. The water content at all levels is at equilibrium. Overdrying virus preparations would tend to upset this balance, removing some of the bound water, causing the oxidation of exposed hydrophilic sites and subsequent denaturation. It is also probable that the slight conformational changes produced by water loss become large, irreversible conformational changes when the virus preparation is incubated at 37 °C, i.e. during the WHO test of thermal stability.

The experimental results show that stable, freeze-dried smallpox vaccines could be produced, with optimal levels of residual water, after much shorter periods of freeze-drying.

The importance of 'maintaining' a high level of –SH groups during freeze-drying and after storage at 37 °C is clearly shown in the Tables. This is supported by the work of Levitt(**6**), who first proposed the theory that the thermal denaturation of many proteins involved the oxidation of thiols to disulphide groups. The formation of intermolecular disulphide bonds would tend to produce irreversible conformational changes resulting in the loss of virus infectivity. There is ample evidence in support of this hypothesis. The inactivation of many thiol enzymes during freeze-drying has been shown to be due to the oxidation of –SH groups at active sites. Freeze-dried human serum albumin, after storage of several months, sometimes exhibits dimerization, and recent experiments at the Lister Institute has indicated that –SH groups are involved in this polymerization of albumin molecules.

In a recent paper Ralston(**8**) has shown that the thermal stability of β-lactoglobulin is dependent on maintaining the integrity of the sulphydryl groups. The mechanism by which peptone prevents the oxidation of –SH groups remains unknown.

But in this laboratory work is continuing on this problem. Preliminary experiments with reduced glutathione and with synthetic polymers containing substituent thiol groups as cryoprotective agents have shown some promise.

SUMMARY

This paper presents evidence that the stability of freeze-dried smallpox vaccine is influenced by the following factors: (*a*) residual water in the vaccine after freeze-drying, (*b*) total nitrogen and (*c*) loss of sulphydryl groups and an increase in disulphide groups. Stability was expressed as loss of virus infectivity after storage at 37 °C. Residual water in the freeze-dried vaccine was measured by a new gas-chromatographic method and this paper includes a brief outline of the technique. Total sulphydryl groups were measured by an amperometric method; disulphide groups were first reduced and titrated as sulphydryl groups.

The results showed that stable vaccines could be produced with an optimal level of residual water after much shorter periods of freeze-drying than those previously used. It was also found that the secondary drying cycle was not required. These findings have enabled this laboratory to raise production by increasing the number of 'freeze-drying runs' in unit time.

When vaccinia virus preparations, derived from chick fibroblast tissue cultures, were freeze-dried there was a considerable loss of sulphydryl groups and an increase in disulphide groups. This was invariably followed by a reduction of virus stability at 37 °C.

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Discussion

EHRENGUT (West Germany) Did you use ascorbinic acid before freeze-drying?

ROBINSON (UK) Ascorbinic acid and cysteine are both inactivators of vaccinia virus as are many –SH compounds. Glutathione, however, is protective.

RICHTER (West Germany) We tested with the Fisher method a lot of vaccines to find the border for tropical conditions at 60 °C. We find the residual-water-border at about 3 %. With more residual water the stability decreases.

ROBINSON (UK) The Karl Fisher reagent is difficult to use and notoriously inaccurate. It works where you want to determine the residual water in bags, but not with small quantities of vaccine.

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INTRACELLULAR AND EXTRACELLULAR FORMS OF POXVIRUS

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A characteristic of poxviruses is that, after replication, over 90% of the virus yield remains within the infected cells. Because of this, most poxvirus preparations, such as those used in neutralization tests or as inactivated vaccines, consist predominantly of intracellular virus that has been released by artificial disruption of cells. However, the virus mainly responsible for the spread of infection through an animal is probably the small amount of extracellular virus that is released naturally from infected cells. Recently it has become evident that these two forms of virus differ in several respects, particularly in the nature of their surface antigens (**1**, **4**, **8**). In this paper we review what is currently known of the differences between extracellular and intracellular poxviruses, and briefly discuss their relevance to immunity against poxviruses.

METHODS

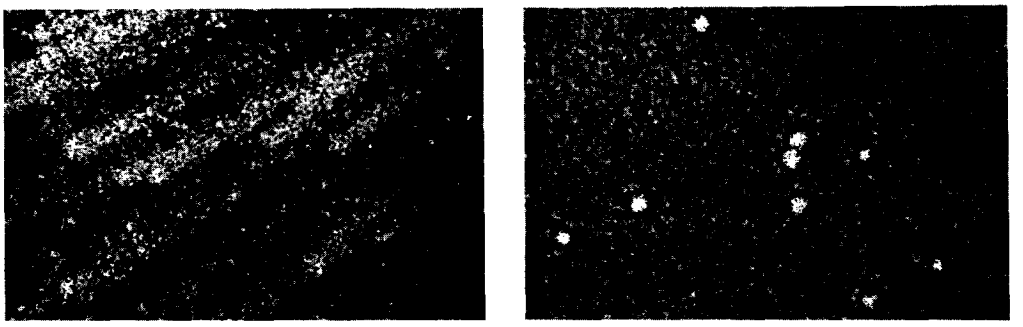
Many of these were described by Appleyard *et al.* (**1**).

Viruses The Utrecht strain of rabbitpox virus was grown in monolayer cultures of HeLa cells. To harvest intracellular virus (ICV) the infected cells were washed, resuspended in fresh medium and disrupted by ultrasonic vibration. To obtain extracellular virus (ECV) the virus growth medium was centrifuged to remove free cells and the supernatant fluid was retained. When ECV was to be used in neutralization tests the medium was supplemented with 0·1% antiserum prepared against inactivated vaccinia virus; this antiserum neutralized a fraction of ECV that behaved as ICV in neutralization tests. Virus infectivity was titrated as pfu in HeLa cell monolayers (**2**).

Antisera Antiserum against live rabbitpox virus (RP antiserum) was obtained from rabbits that had recovered from a rabbitpox infection and then received an intravenous injection of 2×10^8 pfu of virus grown in rabbit testis. Antiserum against inactivated vaccinia virus (KV antiserum) was produced in a horse by five intramuscular injections of about 3 mg inactivated vaccinia virus with Freund's complete adjuvant.

Neutralization tests Samples of virus suspension, containing about 1500 pfu/ml, were added to equal volumes of serial dilutions of the antiserum to be tested and the mixtures were incubated at 36 °C for 6 h. The antibody titre of the antiserum was the reciprocal of the dilution that neutralized 50% of the original virus infectivity.

Electron microscopy Virus samples were mixed with an equal volume of 1% phosphotungstic acid and applied to Formvar-coated specimen grids. After draining and drying the grids were coated with carbon and examined in a Philips EM 300 electron microscope at 80 kV.



(*a*) (*b*)

Fig. 1. HeLa cell monolayers infected with rabbitpox virus. (*a*) Incubated for 3 days under liquid overlay without antiserum. (*b*) Incubated under overlay containing 1/400 RP antiserum.

Density-gradient centrifugation. The virus suspension was mixed with an equal volume of caesium chloride solution, density 1·5 g/ml and centrifuged in the Spinco SW 39 rotor at 38000 rev/min for 16 h. Fractions were taken from the bottom of the tube and tested for virus infectivity by plaque assay and for density by measurement of refractive index.
Fluorescence microscopy The globulin fractions of RP and KV antisera were conjugated with fluorescein isothiocyanate. Infected cell monolayers on coverslips were stained with conjugated antibody either after fixing in acetone, to show internal antigens, or before fixing to show surface antigens. The stained cultures were examined microscopically under dark-ground ultraviolet illumination.

RESULTS

The suppression of virus spread in tissue culture Earlier studies showed that antisera from rabbits recovered from rabbitpox infection (RP antisera) could passively protect normal rabbits against an otherwise fatal dose of rabbitpox virus(**5**). Antisera prepared against inactivated vaccinia virus in Freund's adjuvant (KV antisera), which contained much more neutralizing antibody as measured by conventional methods, had no such protective action(**4**, **6**).

This difference in activity between the two types of antiserum was reproduced in a cell culture system. HeLa cell monolayers were infected with about 100 pfu of rabbitpox virus, overlaid with liquid medium containing actinomycin D 0·02 μg/ml, and incubated for 3 days at 36 °C. Staining with crystal violet showed that virus had spread widely from the initial foci of infection to produce secondary plaques and diffuse, comet-shaped areas of cell destruction (Fig. 1 *a*). The addition of RP antiserum to the liquid overlay prevented this spread of virus and allowed only the development of localized plaques (Fig. 1 *b*). The anti-comet titre of the RP antiserum, defined as the reciprocal of the highest dilution of serum that completely suppressed distant virus spread, was 400. In contrast to the activity of RP antiserum, KV antiserum was inactive in this test even at a dilution of 1/25.

To determine the reason for the inactivity of KV antiserum, the overlay media

Table I. *Virus infectivity in overlay media containing antiserum*

Antiserum in medium	Mean infectivity of four cultures (log pfu/ml)
Nil	5·68 ± 0·14
RP antiserum 1/200	1·41 ± 0·08
KV antiserum 1/200	5·21 ± 0·36

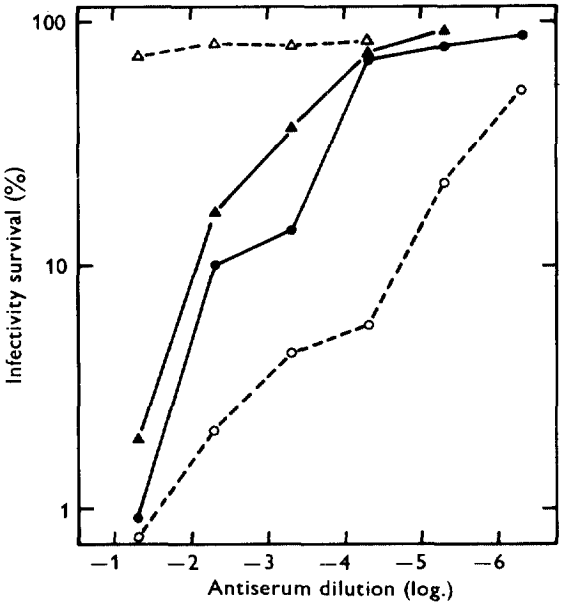


Fig. 2. Neutralization of intracellular and extracellular rabbitpox virus. ●—●, ICV + RP antiserum; ▲—▲, ECV + RP antiserum; ○ – – ○, ICV + KV antiserum; △ – – △, ECV + KV antiserum (Appleyard *et al.* (1)).

from cell cultures used in anti-comet tests were titrated for their content of infective virus (Table I). RP antiserum almost eliminated infective virus from the medium, whereas KV antiserum only reduced the infectivity to about half of that in the absence of antiserum. This result could obviously account for the inability of KV antiserum to prevent the spread of infection. It suggests, further, that the extracellular virus (ECV) present in the overlay medium differed antigenically from the intracellular virus (ICV) against which KV antiserum was active in conventional neutralization tests.

Neutralization of intracellular and extracellular virus

Antisera raised against live rabbitpox virus and inactivated vaccinia virus were titrated for their neutralizing activity against ICV and ECV. Because about

Table II. *Properties of RP antiserum after absorption with intracellular or extracellular virus*

Experiment	Serum absorbed with	Neutralizing antibody titre		Anti-comet titre
		Against ICV	Against ECV	
1	Nothing	56000	2000	400
	ICV	300	1000	400
2	Nothing	16000	6000	400
	ECV	13000	160	< 50

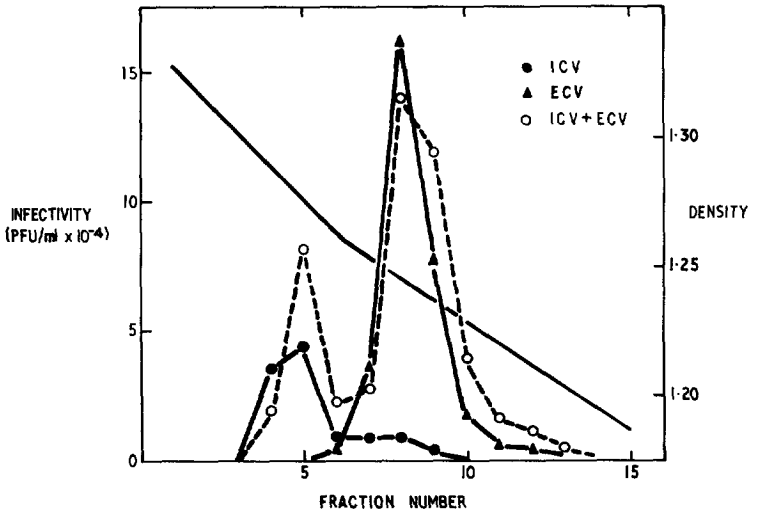


Fig. 3. Equilibrium centrifugation of rabbitpox virus in caesium chloride. A 5 ml suspension was centrifuged at 38000 rev/min for 18 h and 0·33 ml fractions were taken from the bottom of the tube.

50% of ECV resembled ICV in being readily neutralized by KV antiserum, the ECV preparations used in neutralization tests were pretreated with 0·1% KV antiserum as described under Methods. Fig. 2 shows that RP antiserum neutralized both ICV and ECV, the neutralization titres being 13000 and 6000 respectively. In contrast, although KV antiserum had a titre of more than 1000000 against ICV, it had little effect on ECV even at a dilution of 1/10.

Such results indicated that ICV and ECV were neutralized by different antibodies, both types of antibody being present in RP antiserum but only antibody to ICV occurring in KV antiserum. To confirm the existence of two antibodies, attempts were made to remove each one separately from RP antiserum. Table II shows the effects of absorbing RP antiserum by incubation with either ICV or ECV at a ratio of 2×10^{10} pfu virus to 1 ml serum. Absorption of the antiserum

Fig. 4. Extracellular rabbitpox virus stained with phosphotungstate. From Appleyard *et al.*(1).

Fig. 5. HeLa cell culture infected with rabbitpox virus and stained, before fixation, with fluorescein-conjugated RP globulin.

with ICV removed over 99% of its neutralizing activity against ICV without significantly affecting that against ECV. Conversely, absorption with ECV removed activity against ECV but not against ICV. Anti-comet tests on the absorbed sera showed that the ability to prevent virus spread was associated with neutralizing antibody directed against ECV.

Electron microscopy A possible explanation for the antigenic difference between ECV and ICV would be that ECV acquired an additional coat as it was released from the host cells. Negatively stained preparations of the two forms of virus were therefore examined by electron microscopy. Many ECV particles were found to be surrounded by well-defined envelopes (Fig. 4). The envelopes were frequently damaged or incomplete, which might account for the susceptibility of a proportion of ECV to neutralization by KV antiserum. In preparations of ICV, only a few enveloped particles were seen.

Density-gradient centrifugation The presence of a lipoprotein envelope around particles of ECV would be expected to reduce their density compared with that of ICV. Equilibrium density-gradient centrifugation in caesium chloride showed that the buoyant density of ICV was 1·27–1·28 g/ml, whereas the density of ECV was 1·23–1·24 g/ml. This difference was sufficient to permit the separation of the two forms of virus from an artificial mixture (Fig. 3).

Fluorescence microscopy of infected cells If the outer envelope of ECV was derived from the modified host-cell membrane, then the virus antigen that reacts with antibody in RP antiserum should also be present on the surface of infected cells. To test this possibility, cultures of HeLa cells infected 24 h previously with rabbitpox virus were treated, either fixed or unfixed, with fluorescein-conjugated globulin derived from RP or KV antiserum. In fixed cultures the cytoplasmic virus antigen was stained by both types of globulin. Unfixed cultures reacted with RP globulin to produce a bright granular fluorescence on the surface of infected cells (Fig. 5), whereas there was little or no fluorescence when the cells were treated with KV globulin. The surface fluorescence produced by labelled RP globulin could be blocked by prior treatment of the cells with unlabelled RP antiserum, but not by unlabelled KV antiserum. Thus, both types of antiserum reacted with virus antigens inside the cell, but only RP antiserum reacted with a virus-induced antigen on the surface of infected cells.

DISCUSSION

The results described demonstrate the existence of two types of poxvirus neutralizing antibody. Antibody to ICV is formed both as a result of infection and in response to immunization with inactivated vaccine, whereas antibody to ECV is produced only by animals infected with live virus. This conclusion has been amply confirmed by the studies of Turner & Squires(**8**) on vaccinia virus and Baxby(**3**) on vaccinia and cowpox viruses.

A possible basis for the antigenic difference between ECV and ICV would be

the acquisition by ECV of an additional envelope derived from the modified host-cell membrane. Our electron micrographs have shown envelopes around particles of ECV. However, Turner & Squires(**8**) were unable to detect such envelopes on vaccinia virus grown in chick embryo cells. Further morphological studies will be required to resolve this apparent discrepancy. The lower density of ECV, compared with that of ICV, is consistent with its possession of an outer lipid-containing envelope. Although only about 50% of ECV resists neutralization by KV antiserum, all the virus banded at the lower density. This suggests that the neutralizable ECV particles possess damaged envelopes rather than lack envelopes completely.

Anti-comet tests on absorbed RP antisera showed that antibody directed against ECV is more important than antibody against ICV for preventing the spread of infection in cell cultures. This probably applies also to infection in the whole animal and accounts for the protective activity of RP antiserum and the contrasting lack of activity of KV antiserum. Antibody to ICV may have some role in protection against poxvirus infections, but the evidence available at present suggests that it is a minor one. This conclusion has important practical applications. The most immediate is that the usual neutralizing antibody test, using ICV, can give only an indirect measure of the immunity of an individual. In some circumstances, as after immunization with inactivated vaccine, the results could be very misleading. A better index of humoral immunity is likely to be obtained by titrating serum for its neutralizing activity against ECV, or by use of the more convenient but less sensitive anti-comet test.

The results also explain the relative ineffectiveness of inactivated vaccines in protecting animals against rabbitpox infection(**6**, **7**) and suggest that such vaccines would have similar limitations in man. The immunity that is induced by inactivated vaccines, usually sufficient to protect against death although not against disease, is probably not humoral but cell-mediated. It might seem that the ideal vaccine would consist of inactivated ECV. However, Turner & Squires(**8**) were unable to detect neutralizing antibody against either ICV or ECV in the sera of rabbits immunized with inactivated extracellular vaccinia virus, and Appleyard, Hapel & Boulter (unpublished) detected no antibody in similar tests with extracellular rabbitpox virus. In both studies the immunized animals were protected against virus challenge, but possibly by cell-mediated mechanisms. The considerable amounts of ECV neutralizing antibody produced during active infection may be the result of more effective stimulation by the ECV produced under those conditions, or the antibody may be formed in response to the surface antigen of infected cells.

SUMMARY

Poxviruses exist in two antigenically distinct forms – intracellular and extracellular. Extracellular virus differs from intracellular virus in its lower density and the possession of an outer envelope. Studies with fluorescein-labelled antibody suggest that the envelope is derived from the modified surface

membrane of the host cell. Antisera from animals recovered from a poxvirus infection neutralize both forms of virus, but antisera from animals immunized with inactivated virus neutralize only intracellular virus. Only antisera that contain antibody to extracellular virus are able to prevent the spread of infection in cell cultures or animals. Some implications of these findings for poxvirus immunity are discussed.

We wish to thank Mr W. J. Harris for electron microscopy.

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Discussion

HEKKER (The Netherlands) What is the percentage of extracellular and intracellular virus after 2 or 3 days in a completely damaged monolayer?

APPLEYARD (UK) It depends a great deal on the virus you used and the cell you used. With our rabbitpox virus about 2 % comes out, and it is surprising that even if you incubate for a very long time, about 3 days, most of the viruses are still cell-associated. Presumably in the end when everything is completely dead, everything must come out. But most remains inside the cells for a long time.

TAGAYA (Japan) Concerning Dr Hekker's question, I may add that this depends on the cell type which is used. Our experience with HeLa cells and vaccinia virus indicated that up to 48 h p.i. extracellular virus was far less than intracellular virus, but after 72 h p.i. extracellular virus was at the same or a higher level than intracellular virus. I would like to ask you:

(1) What kind of antigen appeared on the surface of infected cells?

(2) Did your purified extracellular virus contain any host cell component?

(3) Have you any experience of neutralizing extracellular virus by anti-intracellular serum in combination of anti-soluble antigen antibody?

Concerning the surface antigen of vaccinia-infected cells, we noticed that at an early phase of infection vaccinia-specific early antigen(s) could be detected on the surface of infected cells, being followed by the appearance of late antigens in the progress of infections. At least some of these antigens recognizable on the surface of infected cells are considered as a part of virus constituent proteins.

APPLEYARD (UK) (1) The antigens detected on the surface of infected cells by fluorescent antibody staining are virus-specified materials. Since antibodies to them are present in RP antiserum but not in KV antiserum, we feel that they are probably the same as the antigen(s) in the envelope of extracellular virus – but there is no direct evidence for this.

(2) We were unable to detect neutralization of extracellular virus by antihost serum. Therefore either (i) extracellular virus has no host component on its surface, or (ii) it does contain a host component, but combination of this component with antibody does not affect virus infectivity.

(3) No, we have not attempted to neutralize extracellular virus with a *combination* of antiserum to intracellular virus and antiserum to poxvirus soluble antigen.

We have found that antisera to soluble antigen, which were previously reported as having high antibody titres against *intra*cellular virus, have little neutralizing activity against *extra*cellular virus. This probably accounts for the relatively poor protective effect of immunization with soluble antigen against virulent rabbitpox infection in rabbits.

EHRENGUT (West Germany) You were referring to the poor quality of inactivated vaccines made out of extracellular virus. In the Vaccination Institute of Hamburg we have produced two forms of non-infectious vaccine, one out of extracellular, the other out of intracellular virus. In clinical practice from a study of the frequency of complications (vaccinial ulcers in primary vaccinees, pre-immunized with killed virus and later vaccinated) we can say that ulcer formation was of identical frequency in individuals immunized with both types of vaccine. Therefore, for the prophylaxis of vaccinial complications, this interesting observation does not seem to be of any help.

APPLEYARD (UK) I am interested to hear of this study, but not surprised at the result. Turner and Squires at the Lister Institute immunized rabbits and mice with inactivated extracellular vaccinia virus, and we made a small study with extracellular rabbitpox virus. Neither preparation elicited neutralizing antibody against either extracellular or intracellular virus, and so were unlikely to be *better* vaccines than inactivated intracellular virus. They did, however, protect against death from virulent challenge virus – as also did inactivated intracellular virus.

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CONTROL OF BACTERIOLOGICALLY STERILE SMALLPOX VACCINE

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With the introduction of a bacteriologically sterile smallpox vaccine prepared in fertile eggs or cell cultures it has now become possible to apply all the modern test methods of control to this vaccine. A suggested method for the control of such vaccines is given in an annexe to this paper.

As with all other virus vaccines, the virus strain must be approved and should be shown to be capable of giving rise to vaccine protecting against the disease without causing any undue complications. The seed virus system must be applied and the seed virus pool shall be shown to be free from extraneous agents. With this vaccine it is also particularly important that a portion of the batch of eggs or tissue culture used for the production of each batch of vaccine shall be set aside as uninoculated controls.

Tests on the supernatant fluids and the tissues of the uninoculated control tissues are extremely important. The supernatant fluids are inoculated into other cell cultures and subcultures are made in order to allow adequate time for a contaminant virus to grow. The tissue is tested for the presence of haemadsorbing viruses using red blood cells from more than one species of animal. Reliance must be placed on such tests in order to show that no contaminating virus or bacteria may have entered the virus harvest during replication in the tissue.

The testing of the virus harvest is giving rise to the greatest number of problems because it is extremely difficult to neutralize all the virus in the virus harvest, especially when there are 10^8 pfu virus particles present. For this reason, tissue culture techniques or inoculation of eggs used for the detection of extraneous agents in the virus harvest are proving extremely difficult to apply. This problem was met also in the control of Rubella vaccine but this was overcome by allowing the addition of further quantities of neutralizing serum at each tissue culture passage in the testing of the vaccine. With smallpox, however, there appears to be an additional problem in that the neutralization is proving even more difficult than was the case with Rubella.

The only tests that can be applied to the virus harvest, as far as we are aware, therefore, is the inoculation of adult mice both intraperitoneally and intracerebrally in order to show that there are no viruses that will kill the mice by these routes of inoculation. Further tests for bacteria, fungi and mycoplasma are possible but we are still unable to incubate tissue cultures in the presence of

neutralized virus/serum mixtures for any length of time without the eventual breakthrough of the un-neutralized virus. It would be extremely useful if a sufficiently potent serum could be prepared such that it would hold back as high as 8 logs of virus throughout a 21-day incubation period so that modern tissue culture techniques could be applied to this vaccine.

The tests applied to the final vaccine are those that have been applied for many years – sterility, innocuity, potency, identity and tests for extraneous proteins are applicable to this vaccine. A test for the presence of extraneous protein is one more generally applied to vaccines today. I have included it more as a point for discussion rather than having any set ideas about the acceptable levels.

It is of course important to check that the vaccine is stable, especially when it may be used in countries where the ambient temperature is relatively high, and it is important also to know that the first five consecutive lots are giving reproducible results, especially with respect to immunity against the disease in man.

It will be extremely important and interesting to keep a large number of subjects, given this new vaccine under surveillance for several years so that the data on the incidence of encephalitis, if any, may be obtained with accuracy. Hopefully, these associated cases, as a result of the use of this new vaccine, will be absent or much reduced. If this is the case then the old-fashioned lymph, as we have known it for over a century, must surely cease to be used.

ANNEX I

Suggested regulations for the manufacture and testing of bacteriologically sterile smallpox vaccine prepared in fertile eggs or on cell cultures

(1) *Definition*

Smallpox virus vaccine consists of a suspension of live vaccinia virus.

(2) *Production and testing facilities*

Smallpox vaccine shall be produced in completely separate areas using separate personnel and equipment. Procedures which involve the presence of micro-organisms other than the vaccine strain or the use of tissue culture cell lines or strains other than that used in the preparation of the vaccine shall not take place in the production areas.

(3) *Virus strains*

(3.1) *Strain* The virus strain used for the production of smallpox vaccine shall be approved by the Licensing Authority. The strain shall be identified by historical records including origin and manipulation if any.

The strain shall have been shown to be safe and antigenically potent in man.

(3.2) *Seed* The production of vaccine shall be based on a seed virus system and the final vaccine shall not represent more than ten passages from the strain culture on which the original laboratory and clinical trials were made.

(3.2.1) *Tests on seed virus for extraneous viruses* The seed virus shall be shown to be free from all extraneous viruses by the methods approved by the Licensing Authority.

(4) *Manufacture of vaccine*

(4.1) *Propagation of virus* Virus shall be propagated in chick embryos or suitable tissue cultures approved by the Licensing Authority.

(4.1.1) *Fertile eggs and tissue cultures* If fertile eggs or chick embryo tissue cultures are used they shall be derived from a healthy flock free from known adventitious agents including *Salmonella pullorum*, *Mycobacterium tuberculosis*, fowl pox, Rous Sarcoma and the fowl leucosis complexes, including neurolymphomatosis. If animal tissue cultures are used they shall also be derived from healthy animals known to be free from the appropriate specific pathogens.

(4.2) *Tests on tissue used for virus growth* When vaccine is prepared in fertile eggs ten eggs from each batch shall be set aside as uninoculated controls. At the time of virus harvest from the inoculated eggs the allantoic fluids from the control eggs shall be pooled and examined for extraneous agents.

When vaccine is prepared on tissue cultures 10 % of each lot of cultures shall be held as uninoculated controls and observed for 14 days beyond the time of inoculation of the production tissue with vaccinia virus. At the time of virus harvest from the production tissue, the fluids from the control shall be examined for the presence of adventitious agents.

(4.2.1) At least 50 ml shall be divided equally between tests made on a suitable monkey kidney, chick embryo, human tissue and a tissue of the same species but not the same animal as that used for production. Culture systems shall be observed for at least 7 days. Subcultures shall be made from the inoculated tissues at 7 days observing each subculture for 7 days.

(4.2.2) For vaccine made in eggs or chick tissue. At least 5·0 ml of the bulk harvest tested in avian tissue shall be used to detect avian leucosis viruses by methods approved by the Licensing Authority. A further 5·0 ml of the bulk harvest tested in avian tissue shall be inoculated on to chick liver cell cultures to detect avian adenovirus.

(4.2.3) For vaccine made in eggs or chick tissue. At least 10 ml shall be divided between (i) inoculation in 0·5 ml quantities into the allantoic cavity and yolk sac in separate groups of embryonated eggs and (ii) inoculation in 0·1 ml quantities on to the chorioallantoic membranes of fertile eggs known to be sensitive to Rous Sarcoma virus.

(4.2.4) For vaccine prepared in eggs the allantoic fluid from the control eggs shall be tested for the presence of haemagglutinating viruses.

(4.2.5) For vaccine prepared on cell cultures at least 25 % of the control tissue shall be examined for haemadsorption viruses using guinea-pig red blood cells as well as those of other species.

The fertile eggs or tissue for virus propagation is satisfactory only if at least 80 % of the controls survive the observation period and all tests show no evidence of virus contamination.

(4.3) *Tests on bulk harvest* Immediately on harvesting the virus and before clarification, samples shall be set aside for examination for extraneous microbial agents. If the virus harvest is not tested immediately it shall be stored at −60 °C or below. Vaccine prepared in eggs shall be processed by a method approved by the Licensing Authority.

(4.3.1) The virus harvest shall have a minimum titre of 10^8 pfu/ml. For the purpose of the following test (4.3.2) the virus harvest shall be neutralized with high titre antiserum of non-human, non-simian, or non-avian origin. The animal used for antiserum production shall be a different species from that used for tissue culture for vaccine production.

(4.3.2) Each of at least ten adult mice shall be inoculated intraperitoneally with 0·5 ml and intracerebrally with 0·03 ml of the original virus harvest and the mice observed for at least 21 days.

(4.3.3) 0·1 ml shall be inoculated on to each of 20 plates of solidified medium and 1·0 ml shall be inoculated into each of four tubes containing 10 ml of liquid medium. Both media shall be shown to be capable of growing mycoplasma under the conditions of cultivation used.

Samples for this test shall be stored at 2–5 °C if held for a period not exceeding 24 h, otherwise the samples shall be frozen at −20 °C or below.

(4.3.4) Bacterial and fungal contamination shall be tested for by appropriate methods.

The virus harvest is satisfactory only if all these tests indicate the absence of extraneous micro-organisms.

(4.4) *Clarification* After harvesting the virus, the suspensions shall be clarified by a method ensuring the removal of all tissue cells.

(5) *Production consistency*

A series of five consecutive vaccine batches shall have been satisfactorily prepared and tested according to these regulations before any vaccine may be released.

(6) *Tests on final containers*

(6.1) *Sterility and innocuity* The vaccine from final containers shall be tested for bacterial, fungal and mycoplasmal sterility as well as innocuity.

(6.2) *Potency and identity tests* The virus shall be identified by a suitable test and shall be titrated in chick chorioallantoic membranes or a suitable tissue culture system using a reference virus preparation. The concentration of live vaccinia virus contained in the vaccine shall be approved by the Licensing Authority. The vaccine shall be shown to be heat-stable by methods approved by the Licensing Authority.

(6.3) *Extraneous protein* If animal serum is used at any stage in the manufacture it shall not be used in such quantity that the final medium will contain more than 1 part of animal serum in 1 million. Any stabilizing agent added to the vaccine shall be approved by the Licensing Authority.

(7) *Samples and protocols*

Samples and protocols for each lot of vaccine shall be submitted to the Licensing Authority.

Discussion

NETTER (France) (1) What do you mean with 'safe seed'; have you in mind postvaccinial encephalitis? (2) What is an immunogenic seed? (3) Why do you allow ten passages in tissue culture; is it proved that it will still protect because for CAM vaccine less passages were allowed?

PERKINS (UK) (1) No: local reactions. (2) We have no animal model; has anybody some other information? (3) In WHO requirements you can find that not more than five or ten passages are allowed. No one has even taken those viruses through 5 or 10 passages.

MARENNIKOVA (USSR) Ten passages seems to me a very high number. We know that during five passages the virus may be changed very much. This requirement should be made more strict. Seed virus has to be checked every two passages.

MURRAY (UK) Because of the impossibility of neutralizing the 'bulk harvest' from cell cultures used for the production of smallpox vaccine, it has been suggested that it may be possible to test samples of the supernatant fluid from the cultures by serial passage in the appropriate test substrates in the presence of a suitable antiserum. We propose to investigate the feasibility of this procedure with a serum supplied by Dr Boulter via Dr Perkins.

EDSALL (UK) I share the hope that we will have generally available around the world a bacteriologically sterile smallpox vaccine in the future. I have never myself known of any evidence that any of the serious reactions after smallpox vaccination have been associated with the small residue of contaminating agents which may be present in otherwise well controlled calf lymph at the present time.

PERKINS (UK) There are no data in the world to say that the removal of the bacterial, fungal or even the commensal bacterial population from this vaccine will reduce the reactions that are known to occur as a result of vaccination. But we always have the attitude in the UK that where it has been shown that a vaccine can be made free from that contaminant, this will be required.

EDSALL (UK) I do not think we should be too hopeful about reducing the incidence of serious reactions.

PERKINS (UK) If this disease disappears from the globe and the vaccine is to be used only for travellers I would demand that the vaccine should be as pure as we could get it.

WEISZ-MALEČEK (Yugoslavia) We control our tissue-culture-produced smallpox vaccine according to the requirements Dr Perkins mentioned. The virus material is diluted 1 : 10 and is inoculated into adult mice (i.c. and i.p.). We are not sure that we neutralized the virus material with antiserum of high titre. Anyhow, the 80% mice survived the inoculation. We will try to get better antiserum on the basis of extracellular and intracellular virus inoculation for neutralization.

PERKINS (UK) A number of people have done this and that is why we could keep this test. This is really one of the few tests at the moment that we can suggest about harvest.

DEKKING (The Netherlands) Which viruses can one detect by inoculation of mice?

PERKINS (UK) The purpose of animal inoculation is not to detect any one virus by a specific test but to use all systems in an attempt to isolate as many viruses as can be detected by tissue culture techniques as well as the use of animals.

EVANS (UK) I entirely agree with Dr Perkins in his appeal for a bacteriologically sterile vaccine and his desire that this should be produced in tissue culture. But we could direct our attention to making bacteriologically sterile lymph vaccine, since this vaccine is now well established with regard to its effectiveness in controlling smallpox, whereas this is not the case with vaccines prepared in tissue cultures.

HENDERSON (WHO) I should like to clarify Dr Perkins's position in regard to his proposal regarding these requirements: whether they might pertain to the UK or be proposed as practicable international standards in the near future. However successful we may be with the eradication programme, I foresee continuing vaccination for at least 5–6 years. Standards such as are proposed require production of either egg- or tissue-culture vaccine, neither of which is a feasible proposition for the developing countries. I should like confirmation that he is speaking only in reference to possible standards for the UK.

PERKINS (UK) I am speaking about the exercise that we are going through in the United Kingdom. The particular vaccine used in a country must remain the responsibility of the national health authority. It will be many years before requirements for a bacteriologically sterile smallpox vaccine can be published and enforced.

LANE (USA) Comparative studies of vaccination complications for new vaccines are not practical given the current low levels of complications. Studies of the efficacy of newer vaccines, using the human smallpox case as an end-point, are already not possible. We do not have standard and time-tested laboratory tests of safety and efficacy. I would therefore like to predict that we will eradicate smallpox using current vaccines *before* we successfully develop a new vaccine, a new production method, or a new set of established laboratory evaluation methods.

DICK (UK) As you know, I agree with what David Evans has said. I cannot see why you cannot clean our presently available calf lymph and, by neutralizing the vaccinia virus test it in tissue culture for extraneous agents. I do not see why you must go over to tissue culture of CAM vaccine before you start trying to test for and get rid of any undesirable agents which may be present. You can get rid of weeds without digging and sowing a new lawn.

HEKKER (The Netherlands) A tissue culture vaccine produced in the way we try to do is much easier to produce than calf lymph. That is one of the reasons for this type of vaccine.

VALLE (Finland) As a comment to the proposal to use a control test where the virus is neutralized by specific antiserum, the fact that there is a non-neutralizable virus fraction of 10–20 % in the present vaccines will decrease the value of this control method in the control of this viral vaccine.

EHRENGUT (West Germany) I am more favourable than Dr Evans regarding the value of tissue-culture vaccine. In Hamburg, for 12 years now, over 350000 individuals have been immunized with tissue-culture vaccine and we already vaccinate 12-year-old children with this vaccine without having more severe complications than using dermovaccine.

EDSALL (UK) The Massachusetts Laboratory has for the past 20 years made calf vaccine which is bacteriologically sterile at least half the time. As regards the protective potency of CAM vaccine, this has been confirmed repeatedly by its use to control smallpox outbreaks in Texas.

HOCHSTEIN-MINTZEL (West Germany) (1) Low bacterial counts in calf skin vaccine does not mean that the bacteria are not there; they are simply killed but not eliminated. (2) There is no theoretical reason why vaccine produced in tissue culture should not be as immunogenic as vaccine produced on calf skin.

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INTERNATIONAL REFERENCE PREPARATION OF SMALLPOX VACCINE

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Work towards the establishment of an international reference preparation of smallpox vaccine is recorded from the mid-1950s. The earliest studies were of two dried vaccines which were investigated as regards their thermostability. One was found to be a stable vaccine but the other deteriorated rapidly at the higher temperatures. It is interesting to note that in these studies the two vaccines were held for varying periods at different temperatures and then tested as regards their vaccination take-rates. It was only on this basis that subsequent comparisons with laboratory results were made using rabbit-skin scarification and CAM pock counts. It was found that those tests ran generally parallel with the vaccination success rates. Even at that time the pock count was found to be a more accurate method of laboratory titration than the other. Samples of seven other batches of vaccine prepared in the same way as the more stable of the two dried vaccines were also tested and the results showed that the stability of the particular dried vaccine was not an abnormal feature.

It was, however, the 10th Expert Committee on Biological Standardization (meeting in 1956) which noted the offer by the Lister Institute of Preventive Medicine, London, of a sample of stable freeze-dried smallpox vaccine prepared in sheep, for an international reference preparation. The Expert Committee asked the Statens Seruminstitut, Copenhagen, to arrange a collaborative study of the material. The following year the 11th Expert Committee was informed that arrangements had been made for this collaborative study; different freeze-dried smallpox vaccines were to be compared, including the proposed international reference preparation. Various assay methods were to be used; in fact, one of the members of the Expert Committee had developed an improved design for the assay of potency of smallpox vaccine by intracutaneous injection into rabbits. The use of tissue culture methods was also mentioned.

It was in the late 1950s also that the Biological Standardization programme of WHO undertook the formulation of sets of International Requirements for Biological Substances. The 12th Expert Committee on Biological Standardization (meeting in 1958) noted that a Study Group was to be convened to draft Requirements for Smallpox Vaccine. The Expert Committee asked the Statens Seruminstitut to include any methods of potency determination which would be recommended by the Study Group in the collaborative assay of the proposed international reference preparation.

The Study Group on Requirements for Smallpox Vaccine met in November 1958, and these Requirements were published in 1959 (4). Certain important considerations were pointed out by the Study Group. They were of the opinion that the only laboratory test for which there had been, at that time, adequate comparison with take-rates in vaccination of humans were the rabbit scarification tests and potency tests in chick embryos. No other methods were therefore included in the Requirements at that stage.

The potency tests using chick embryos included both the enumeration of pockcount units as well as the determination of LD 50 on application of vaccine to the chorioallantoic membrane. It is interesting that it was reported to the 13th Expert Committee on Biological Standardization (which met in 1959) by Dr C. Kaplan of the Lister Institute that an analysis of 23 paired titrations of vaccinia virus by pockcounting and intravenous injection of chick enbryos showed good correlation in reproducibility of titres. Intravenous injection of embryos, however, was not thought to be useful for the assay of vaccines because it was very time-consuming and because of lack of information on the effect of differences in virus virulence on the evaluation of efficacy of the vaccines.

The progress of the international collaborative assay was reported to the 14th Expert Committee on Biological Standardization (meeting in 1960) and the full report was presented to the 15th Expert Committee which met in December 1962. The period, therefore, which elapsed between the offer of the preparation to serve as an international reference preparation and its establishment as such was from 1956 to 1962, a period of 6 years (1).

THE INTERNATIONAL COLLABORATIVE ASSAY

Seven laboratories in as many countries participated in the international collaborative assay. A total of 22 assay results were collected between August 1959 and July 1960. All participants performed the scarification test in rabbits; five laboratories, in addition, arranged a revaccination study in humans, four laboratories used the pockcount in eggs and three laboratories used various other testing methods, including intracutaneous tests in the skin of rabbits, LD 50 of the vaccine of the chorioallantoic membrane of chick embryos and in newborn mice, plaque count in tissue culture and LD 50 in tissue culture. A somewhat condensed report of the collaborative assay as well as a description of the statistical methods used have been published in the Bulletin of the World Health Organization (2, 5). More details, however, were included in the unpublished working documents relating to this study.

Five vaccines were assayed. They were all in the freeze-dried form and the samples were coded. They included the following:

(1) A sheep vaccine from the Lister Institute issued as two samples under different code letters. The origin of this vaccine was legendary and it was supposed to have been derived from a strain of variola isolated in the Prussian army in 1870, obtained from Cologne. It had been used by the British Government Lymph Laboratory and passed in calves until the late 1940s. It had since been passed alternately in rabbits and sheep; the sample assayed was from a sheep passage.

(2) A calf vaccine prepared from a strain of the National Institute of Hygiene, Ecuador, and sent to Copenhagen, where the vaccine was made.

(3) Two egg vaccines, one from a strain used in Berne for smallpox vaccine production and obtained at the turn of the century from an Institute in Lancy, near Geneva, and maintained by repeated passage in cows. The other egg vaccine was from the Mechnikov Institute, Moscow, from a strain used for smallpox vaccine production in chick embryos. It had been maintained by alternate passage in calves and rabbits.

Each of the participants was also asked to include a local vaccine. Those included were one from Turkey (the strain, which had been used from 1935, had been maintained by alternate passage in calves and donkeys), one from Thailand (from a strain obtained in 1905 from the Philippines), one from Denmark (from a strain used for smallpox vaccine production and obtained about 60 years ago from Germany), and one from Tunisia (from material obtained in 1955 and maintained by calf passage). Three other vaccines, from Switzerland, the United Kingdom and the Soviet Union, were from similar material to that included in the coded vaccines sent out for the collaborative assay. It is not necessarily the case that all of the vaccinia strains used for production of these vaccines were derived from cowpox.

Although at the time of the international collaborative assay a number of tests of virus concentration were used, the position at the present time is that reliance is placed on the enumeration of pock-forming units on the chorioallantoic membrane of chick embryos. A detailed description of the findings of the international collaborative assay, therefore, need not be repeated. For anyone interested, the information in the unpublished working document could be made available. For the present, a summary of the findings, particularly in relation to the vaccine established as the international reference preparation, may be of interest.

All of the seven laboratories ranked the coded vaccines in the same order using the scarification tests in rabbits, but three of the four laboratories had the same general result using pock count in eggs.

The results of the scarification test in rabbits and the pock count in eggs were in good agreement, but the variation in the results was greater in the rabbit tests.

The proposed international reference preparation gave an average result of $10^{8\cdot4}$ pfu per ml of vaccine. The range of relative potencies was smaller than the range of titres.

The standard error of the pock-count test was lower than that by the scarification test; differences between vaccines demonstrable by using, say, six eggs for each of five serial dilutions (in threefold steps) could be demonstrated in rabbits only by using a large number of animals.

The revaccination studies in humans made by five laboratories included all five distributed vaccines as well as the local vaccines. Groups of 20–100 persons were used, each person receiving two vaccines, one on each arm. The observations were made according to a common pattern and the results evaluated statistically. The revaccination take-rate corresponded roughly with the interval between the vaccination and the previous vaccination, according to the available information. This ranged from 20% for an interval of about 6–8 years to 60% where the interval was 14–16 years. This, however, was shown in two out of the five laboratories; the remaining laboratories had some variable results. This could have been due to several factors – different vaccines used on the two occasions, inaccurate information on previous vaccination and possible exposure to smallpox in one country.

It should be noted that this international collaborative assay was the first made during which the international requirements for the substance were already available. The collaborative assay also included vaccines, all of which were tested in man in addition to laboratory tests. The proposed international reference preparation was judged to have satisfied the international requirements, was suitable for use in the tests of virus concentration specified therein and accelerated degradation tests had shown that the material was highly stable. The Expert Committee on Biological Standardization established the international reference preparation on this basis.

SOME PROBLEMS IN THE USE OF THE INTERNATIONAL REFERENCE PREPARATION IN THE CONTROL OF SMALLPOX VACCINES

(1) A problem concerning the use of the International Reference Preparation arose from a statement based on an observation by Slonin (which had also been found by others) on the volume of inoculum used on the chorioallantoic membrane for pock counting. It was pointed out that a volume of 0·1 ml gave a higher pock count when the results were calculated than if 0·2 ml were used in this test. Dr Krag, Director of the International Laboratory for Biological Standards at the Statens Seruminstitut, Copenhagen, who was responsible for co-ordinating the international collaborative assay, was able to check with the four participants who had used the pock count in eggs in the international collaborative assay. This inquiry was also verified by communication with these four laboratories. In all four cases the volume of 0·1 ml was used. Specifications for a minimum pock count for acceptable smallpox vaccines should therefore take this into consideration. Unless it could be shown that there is no difference in the results when the two different volumes are used, specifications should preferably indicate that a volume of 0·1 ml should be applied to the membranes.

As already mentioned, the Requirements for Smallpox Vaccine were first formulated by the Study Group which met in 1958(**4**). A WHO Expert Group was convened in 1965 in order to revise four of the sets of Requirements that had been published up to that time; these included smallpox vaccine. The revised Requirements for Smallpox Vaccine were published in 1966(**3**). There were several important changes in the new Requirements based on advances in knowledge and experience. In general, the new provisions tended to be stricter and to give a better assurance of efficacy and safety of the vaccine as compared to the Requirements originally formulated.

As regards the test for virus concentration, the Expert Group, in the new Requirements, stressed the use of pock counts on the chorioallantoic membrane of chick embryos. They were of the opinion that it is only with this test that the results have been adequately correlated with the results of vaccination and revaccination in man. It was, however, also stated in the revised Requirements

that if other tests of virus concentration were used, such as skin scarification in rabbits, the results should be correlated with those obtained using the membranes of chick embryos.

The revised Requirements also specified a higher pock count, namely 1×10^8 pfu per ml of undiluted vaccine, in contrast with the earlier requirement in which the amount specified was 5×10^7 pfu. A more strict specification for the stability of the vaccine was also made. The existing International Reference Preparation, even with these revised Requirements, would continue to be suitable since in the international collaborative studies the pock count exceeded the minimum specification and, in check tests, had also shown adequate stability.

(2) Another problem arose from a complaint that the specifications for heat-resistance of dried vaccines are ambiguous. Such a test, of course, should be made in parallel with a suitable reference preparation. What is stated is that the vaccine passes the test if the requirement for minimum virus concentration is fulfilled and at least one-tenth of the virus concentration is retained after incubation at not less than 35 °C for 4 weeks or more. The ambiguity seems to lie in the question of what the minimum virus concentration should be at the conclusion of the heat-incubation period. Whatever may be stated in these Requirements, it was the intention of the WHO Expert Group which adopted these Requirements that the minimum virus concentration, namely 10^8 pfu, must be fulfilled after the incubation. *In addition,* at least one-tenth of the virus concentration should have been retained. In future revisions of these Requirements it would be necessary to clarify this question. Both aspects of heat-resistance were considered important, particularly in the case of tissue-culture vaccines, which may have a high pock-forming unit count but be relatively less stable than skin vaccines.

(3) Since the international reference preparation has been shown to fulfil these provisions, it has been suggested that the minimum requirements for virus concentration could be modified. This could be without the necessity for specifying an actual pock count but should state that such a count on the test vaccine, under the conditions of the prescribed test, should give a result equal to, or better than, the international reference preparation or an equivalent national reference preparation. This, too, is a point for future consideration in requirements.

SUMMARY

The international collaborative studies leading to the establishment of the International Reference Preparation of Smallpox Vaccine in 1962 are briefly described. Some problems in the use of this international reference preparation and the control of smallpox vaccines in regard to the virus concentration of the vaccines will be mentioned.

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Discussion

Marennikova (USSR) What is the position today 10 years after the establishment of a reference preparation in respect to its potency?

Outschoorn (WHO) I hope some of the participants have these data of reference preparation titrations.

Perkins (UK) I am glad that Dr Outschoorn raised the question of the ambiguity of the test for heat-stability because the European Pharmacopoea appears to have misinterpreted this WHO requirement. If it is the general feeling of the meeting that 10^8 pfu shall remain at the end of the test for heat-stability then we must alter this requirement in the European Pharmacopoea.

Hekker (The Netherlands) I would like to advocate not only the use of the reference preparation in the laboratory but also the reporting of the results in publications so that the interpretation of the titres, whatever method is used, is possible.

Evans (UK) I think we should be quite clear with Dr Outschoorn what was suggested with regard to the alteration of the potency requirement in the WHO International Requirements. Does he suggest that a revised version should read as follows: 'A vaccine should be tested for infectivity in relation to the International Reference Preparation of Smallpox vaccine. The vaccine passes the test if it shows an infectivity equal to or greater than the Reference Vaccine. Such a test may be made in fertile eggs or in tissue cultures'?

Outschoorn (WHO) Yes.

Richter (West Germany) With the differential centrifugation after prepurification with Frigen the addition of antibiotics can finally be removed by centrifugation. It is also possible on the removal of the supernatant after the second step of centrifugation to wash, once or twice, the virus pellet and then antibiotics are surely removed. But the experts who make the requirements do not allow the use of antibiotics. Under the circumstances mentioned the antibiotics are merely eluated. My question is: is it possible to use antibiotics?

SESSION IV

FIELD STUDIES

Chairman: Professor G. W. A. Dick (UK)
Secretary: Dr B. Hofman (The Netherlands)

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POTENCY AND STABILITY CHARACTERISTICS OF SMALLPOX VACCINE USED IN THE SMALLPOX ERADICATION PROGRAMME IN WESTERN AND WEST-CENTRAL AFRICA

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SMALLPOX VACCINE

Wyeth's smallpox vaccine (Dryvax®) used in the African programme is a lyophilized calf-lymph product made from the 22nd to 28th heifer passage of the New York City Department of Health Strain of vaccinia virus and contains peptone as a stabilizing agent.

Since April 1971 the USPHS requirements have included the counting of pocks formed on the chorioallantoic membrane of embryonated chicken eggs (CAM assay) as an alternate to the rabbit scarification (RS) assay procedure. The USPHS potency requirement for the product specifies that the CAM assay titre be at least equivalent to the Reference Vaccine in a simultaneous assay. The WHO recommends a titre in excess of 8·0 ^{10}log pock forming units (pfu's) per ml.

CAM ASSAY

Vaccine produced for use in Africa was tested for potency by the RS and CAM assays. The latter was standardized to the use of 12-day-old embryonated eggs inoculated with 0·1 ml of both production and Reference Vaccines using 8–10 eggs per dilution.

The mean titre of the Reference Vaccine based on 399 CAM assays over $5\frac{1}{2}$ years was 8·3 ^{10}log pfu's per ml with a standard deviation of 0·13. An assay was considered valid if the titre of the Reference Vaccine was not less than 8·0 and not more than 8·6 ^{10}log pfu's per ml. Production lots of vaccine were considered equivalent to the Reference if the titre was not less than 8·0 ^{10}log pfu's per ml.

Vaccine rehydrated for administration by jet gun is a 33-fold (1·5 ^{10}log) dilution of vaccine intended for administration with a bifurcated needle.

During the initial phase of utilizing the CAM assay procedure, we observed that the source of embryonated eggs, the age of embryo and the volume of inoculum affected the assay titre.

The CAM assay titres of the Reference Vaccine obtained with 12-day-old embryonated

eggs from different flocks from one supplier appeared to be 0·6–0·8 ^{10}log lower than the titres obtained with eggs from another supplier or with eggs from Wyeth's RIF-free flock.

In ascertaining the influence of age of the embryo on the infectivity titre of the Reference Vaccine, it was found that the titres obtained with 10- and 11-day-old eggs were respectively 1·0 and 0·5 ^{10}log lower than the titres obtained with 12-day-old eggs. Titres obtained with 13- and 14-day-old eggs were 0·2 $\log_{10}$ higher as compared with 12-day-old eggs. On the basis of standard deviation calculations it was concluded that 11-day-old embryos are not as uniform in sensitivity as older aged embryos.

In a series of assays of the Reference and production lots of vaccine using inoculum volumes of 0·1 and 0·2 ml the infectivity titres obtained with the smaller inoculum were consistently 0·1–0·3 ^{10}log higher. The finding of Slonim *et al.* (2) that higher titre values were obtained with a 0·025 ml inoculum than with 0·1, 0·2 or 0·4 ml was confirmed in our studies.

The combined effect of the volume of inoculum and age of embryonated eggs on the titre of the Reference Vaccine is such that the highest average titre was obtained in 13-day-old embryos using 0·1 ml inoculum whereas the lowest was obtained in 11-day-old embryos with an inoculum of 0·2 ml; the difference was 0·9 ^{10}log.

From the data presented it can be concluded that the use of a reference vaccine for each titration and the establishment of criteria of test validity based on a standardized procedure permit a better estimate of the potency and stability characteristics of smallpox vaccine.

CLINICAL STUDIES

We recognized a need to relate the potency of the US Reference Smallpox Vaccine Lot 2 to performance in man and elected to compare the skin response of young adult revaccinees to the rabbit scarification test ratio and the CAM assay titre of undiluted and diluted preparations of the Reference Vaccine.

Espmark (1) established that less infectious virus is required to obtain a major reaction in primary vaccinees than in revaccinees and that the concentration of virus required to produce a major reaction in revaccinees varied inversely with the interval since their last vaccination. Therefore, administration of vaccine to young adult revaccinees would be a stringent test for potency.

Our study population was from a correctional institution for males 15–22 years of age. A smallpox vaccination is required for all incoming citizens including those entering the institution for a second or third time. Some of the older inmates had been in military service and received a smallpox vaccination at the time of induction. Virtually all had received a primary vaccination between 1 and 6 years of age. For approximately one-half of the population the estimated time interval since their previous vaccination ranged from 10 to 20 years. To control this variable, each individual was administered two vaccinations by an experienced vaccinator (MZB), a test preparation on one arm and undiluted Reference Vaccine on the other arm as the control. The vaccine was administered with the bifurcated needle using 15–20 tangential pressures.

Two coded samples of rehydrated Reference Vaccine were prepared on the day of immunization; the undiluted Reference Vaccine and the other, either a dilution of the Reference Vaccine or an undiluted preparation of the same. The last two preparations are identified as test vaccine whereas the first one is identified as the control vaccine. The vaccinator was not aware of the identity of the samples at time of vaccination or examination of arms 1 week later. The reactions were recorded as either major or equivocal as

Table I. *Responses of young adult revaccinees to vaccination with undiluted and diluted preparations of US Reference Smallpox Vaccine, lot 2, administered with Wyeth's bifurcated needle*

Dilution of test vaccine	No. of trials	No. of vaccinees	No. major reactions on arms that received: Control* vaccine (A)	Test vaccine (B)	Ratio B/A: Average	Ratio B/A: Range in trials
Undiluted	4	87	53 (61 %)†	50	0·94	0·82–1·00
1/2·5	2	27	18 (67 %)	17	0·96	0·93–1·00
1/3·5	2	47	26 (55 %)	25	0·96	0·92–1·00
1/5·0	6	153	99 (65 %)	84	0·85	0·64–1·11
1/10	3	68	43 (65 %)	37	0·85	0·78–0·93
1/100	1	20	15 (75 %)	3	0·2	
Total	18	402	254 (63 %)			

* Undiluted US Reference Smallpox Vaccine, lot 2.

† Percentage of individual developing major reactions on arm that received control vaccine.

defined by the WHO Expert Committee on Smallpox(3) and tabulated as the number of individuals that developed major reactions with each of the coded vaccine preparations. The results as shown in Table I were also converted to a ratio of the percentage of individuals who developed a major reaction on the arm that received the test vaccine (diluted or undiluted Reference Vaccine) to the percentage of individuals having major reactions on the arm that received the control, undiluted Reference Vaccine.

The susceptibility of the 18 groups vaccinated over a period of 15 months were similar as indicated by the percentage of individuals that developed major reactions with the control vaccine (undiluted Reference Vaccine). Sixty-three per cent of the total population developed a major reaction following administration of the control vaccine, and with the exception of the group of 20 individuals that received the 1/100 dilution of test vaccine the response of the other groups did not vary more than 8 % from that of the total.

Of the 87 individuals that received undiluted Reference Vaccine on both arms, there was 94 % agreement in the formation of major reactions at the two sites; 53 individuals developed major reactions on one arm and 50 had a similar response on the other arm. In the four individual trials comprising the undiluted group there was 82 %, 96 % and 100 % (two instances) agreement. The individuals that received either 1/2·5 or 1/3·5 dilution of vaccine on one arm and control vaccine on the other experienced the same frequency of major reactions on both arms. Although the average ratio of test vaccine to control vaccine responses for the groups administered the 1/5 and 1/10 dilutions of vaccine were within the range observed with the group that received the control preparation on both arms, there was one trial in each group that had ratios of 0·64 and 0·78

respectively. The marked difference in the responses obtained with the 1/100 dilution of Reference Vaccine indicated that there was no need to carry out further observations.

Aliquots of the test and control preparations were tested for potency by the rabbit scarification and CAM assays as coded samples. These potency test data and responses obtained in revaccinees indicate that vaccine with a titre of 7·7 ^{10}log pfu's per ml or a rabbit scarification test ratio of 0·5 produces the same frequency of major reactions in young adult revaccinees as the US Reference Smallpox Vaccine lot that has a CAM assay titre of 8·3 ^{10}log pfu's per ml, when administered by multiple pressure technic with the bifurcated needle.

STABILITY STUDIES

One of our early studies concerning the stability characteristics of lyophilized vaccine consisted of an evaluation of the two types of vaccine, i.e. for administration by jet gun or by bifurcated needle. Samples of 11 lots of vaccine stored at 100, 70, 50, 37, 25, 5 and −20 °C were assayed at various time intervals in primary *Cercopithecius* monkey kidney (CMK) cell cultures.

On the basis of 22 assays of the Reference Vaccine, the 95% confidence limits of a smallpox vaccine assay titre by the CMK cell culture system are ±0·4 ^{10}log TCID 50 per ml. The observations obtained for each of the 11 lots in this study were similar. It appeared that significant reduction in the titre of vaccine (0·5 ^{10}log TCID 50 per ml) occurred following 1 h storage at 100 °C, 1 day at 70 °C, 2 weeks at 50 °C, 6 months at 37 °C and 21 months at 25 °C.

In our laboratory, the infectivity titre of smallpox vaccine obtained by the CMK cell culture system expressed as ^{10}log TCID 50's per ml is comparable to the titre values obtained by the CAM assay. The correlation of the two assay systems is presented in Fig. 1. However, this may not be a universal finding since comparable agreement was not obtained in assaying the International Reference Standard by the two systems. The observed differences in titres as shown in Table II may be related to the strain of virus used to prepare the different references. The US and Wyeth References were made with the NYC Board of Health strain whereas the International Reference was prepared with the Lister strain.

A second study was undertaken to establish the stability characteristics of Wyeth's lyophilized vaccine with the CAM assay procedure. The data presented in Fig. 2 describes the average change in titre of six lots of vaccine stored at 37, 25 and 5 °C as compared to the titre of vaccine stored at −20 °C assayed at the same time intervals. The titres of vaccine stored at 25 °C showed a gradual decrease in titre of 0·3–0·4 ^{10}log pfu's per ml. Following 3 months of storage at 37 °C the potency was reduced 0·4 ^{10}log and after 6 months the titre was 0·5 ^{10}log lower than the control vaccines. These findings are quite similar to those obtained with the CMK cell system assays and confirm the interchangeability of the two assay systems.

Long-term stability data for vaccine stored at 2–8 °C were obtained by assay-

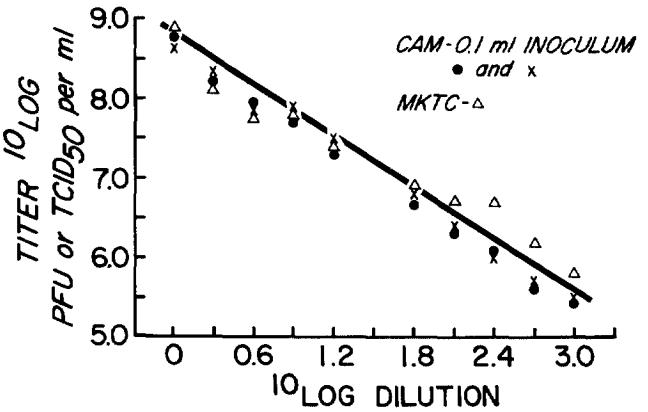
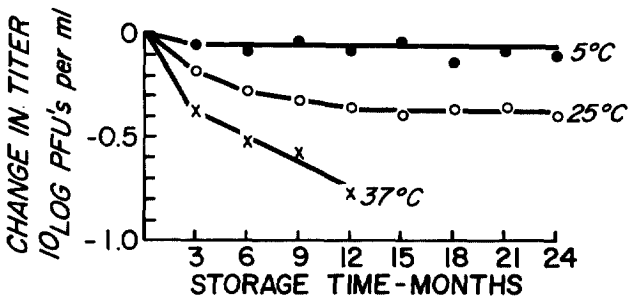


Fig. 1. Comparison of CMK tissue cell and CAM assays of serial twofold dilutions of smallpox vaccine.



*Average response of 6 lots of vaccine for administration by jet gun.

DRYVAX® – Lyophilized smallpox vaccine

Fig. 2. Dryvax® stability study* – lyophilized vaccine stored at 5, 25 and 37 °C CAM assay.

Table II. *CAM and CMK tissue cell culture assay of the international and US Reference Smallpox Vaccines*

Reference	CAM assay ^{10}log pfu's/ml	CMK assay ^{10}log $TCID_{50}$/ml	Titre ratio CMK/CAM
International reference	8·3	7·4	−0·9
US reference Smallpox Vaccine, lot 2	8·4	8·8	+0·4
Wyeth Reference Vaccine	7·9	8·2	+0·3

Table III. *Rehydrated Dryvax® stability study – CAM Assay Titres (^{10}log pfu's per ml)*

Storage time (weeks)	Storage conditions*				US Reference Smallpox Vaccine, lot 2
	A	B	C	D	
0	8·4	—	—	—	8·2
4	8·4	8·3	8·4	8·5	8·3
8	8·4	8·2	8·3	8·4	8·3
12	8·2	8·2	8·2	8·4	8·3
16	8·2	8·1	8·2	8·4	8·2

Dryvax® : lyophilized smallpox vaccine.

* Storage conditions: A, 2–8 °C only; B, 2–8 °C with two 1 h intervals at 25 °C per week; C, 2–8 °C with 20 min intervals at 25 °C 5 days per week; D, at −10 to −20 °C only.

ing samples of 27 production lots stored for 1½–8½ years. The seven oldest lots were, at the time of manufacture, tested for potency by the rabbit scarification test. These lots were first assayed in embryonated eggs in 1968 and again 3 years later. The CAM assay titre of the other 20 lots at time of manufacture ranged from 8·0 to 8·6 ^{10}log pfu's per ml. The titres of the seven oldest lots when assayed in 1968 ranged from 8·0 to 8·3 ^{10}log pfu's per ml. It was evident that lyophilized vaccine may be stored as long as 8½ years at 2–8 °C without significant loss of potency.

A study was undertaken to define the stability characteristics of vaccine rehydrated for use with the bifurcated needle. The study was designed to simulate use of the product in a physician's office or immunization clinic, where it may be exposed to ambient temperatures for various time intervals until the material is depleted. Samples of six lots of rehydrated vaccine were stored at

(A) 2–8 °C only.

(B) 2–8 °C with two 1 h intervals at 25 °C per week for the initial 2 months and for 1 h at 25 °C per week during the last 2 months. Total time at 25 °C was 24 h in 16 weeks.

(C) 2–8 °C with 20 min at 25 °C 5 days per week. Total time at 25 °C was 26 h in 16 weeks.

(D) −10 to −20 °C only.

All vaccines were assayed at 4-week intervals. Since the titres of the six lots at time of manufacture were almost identical (8·3, 8·4, 8·3, 8·5, 8·4 and 8·5 ^{10}log pfu's/ml) the data have been consolidated and are presented as average values in Table III. Vaccines stored under the four experimental conditions maintained potency titres equivalent to the Reference Vaccine for 16 weeks. Some of the vaccines were administered as coded samples to young adult revaccinees with a bifurcated needle. A coded sample of Reference Vaccine was administered to the other arm as previously described. As shown in Table IV,

Table IV. *Skin responses in young adult revaccinees administered* rehydrated Dryvax® stored at 2–8 °C with Wyeth's bifurcated needle*

Storage time (weeks)	CAM assay titre (^{10}log pfu's/ml)	No. of vaccinees	Major reactions (%)				Ratio B/A
			Ref. vaccine A†		Test vaccine B		
			No.	%	No.	%	
0	8·4	86	62	72	59	69	0·96
4	8·1	60	37	62	34	57	0·92
8	8·1	33	22	67	24	73	1·09
10	8·1	29	24	83	22	76	0·92
12	8·2	49	39	80	34	69	0·87
13	7·9	73	46	63	41	56	0·89
15	8·0	21	13	62	13	62	1·00
16	8·0	36	23	64	23	64	1·00
17	8·2	30	20	67	19	63	0·94

Dryvax®: lyophilized smallpox vaccine.
* Storage condition B, see text.
† US Reference Smallpox Vaccine, lot 2 – 8·4 ^{10}log pfu's per ml.

Table V. *Stability characteristics of smallpox vaccine rehydrated for administration with a jet gun*

Storage		Change in titre (^{10}log TCID 50/ml)* from zero time	
Temperature	Time (h)	Diluent without phenol	Diluent with 0·25 % phenol
5 °C	8	0·0	−0·1
	24	−0·1	−0·1
	32	0·0	−0·1
25 °C	8	0·0	−0·1
	24	0·0	−0·2
	32	−0·1	−0·2
37 °C	8	0·0	−0·2
	24	0·0	−0·3
	32	0·0	−0·3
18 h at 37 °C + 6 h at 25 °C		0·0	−0·3

* Combined data of 3 studies using *Cercopithecus* monkey kidney tissue cell cultures.

the ratio of the percentage of major reactions that developed following administration of test vaccine (B) to the percentage obtained with Reference Vaccine (A) ranged from 0·87 to 1·09 during the 17 weeks of observation. At 15-, 16- and 17-week intervals the ratios were 1·0, 1·0 and 0·94 respectively, confirming the laboratory assays that Wyeth's smallpox vaccine maintains adequate potency following rehydration and storage at 2–8 °C for 4 months.

Table V summarizes the results of three studies establishing the stability characteristics of vaccine rehydrated for administration by jet gun. The study evaluated the use of diluent without preservative which was supplied for use in Africa, and diluent containing 0·25% phenol. Vaccine rehydrated with diluent without phenol did not lose titre after storage at 37 °C for 32 h. Vaccine rehydrated with phenol containing diluent showed a 0·3 $\log_{10}$ reduction in titre during the same time interval.

SUMMARY

We have presented evidence indicating that in assaying the potency of smallpox vaccine by the CAM procedure, lower titre values may be obtained depending upon the source of embryonated eggs, the age of the embryos, and volume of the inoculum. For this reason, it is desirable to standardize the assay with use of 12- to 13-day-old embryos, an inoculum of 0·1 or 0·05 ml and simultaneous titration of a reference vaccine to evaluate the sensitivity of the assay.

The WHO and USPHS Smallpox Vaccine requirements have at least a two- to fourfold excess potency factor. This is indicated by our observations that vaccine with a titre of 7·7 ^{10}log pfu's per ml will produce the same frequency of major reactions in young adult revaccinees as vaccine with a titre of 8·3 ^{10}log pfu's per ml. The excess potency factor is surely greater in the case of primary vaccinees.

The successful smallpox eradication programme in a segment of Africa corroborates the results of stability studies indicating that lyophilized and rehydrated vaccines would maintain adequate potency when stored and used as directed.

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Discussion

EHRENGUT (West Germany) We made a study, similar to Dr Bernstein's, on medical students. The conventional vaccine was diluted 5 times, and was applied to one arm of the individual, on the other arm we applied the conventional vaccine (10^8 pfu). The results were similar to those of Dr Bernstein.

ROBINSON (UK) What method did you use to estimate the residual phenol after freeze-drying and how much phenol do you find? At the Lister Institute we find that almost all phenol is removed during freeze-drying and appears in the condenser.

BERNSTEIN (USA) Phenol is present in the lyophilized pellet at a level of 0·10–0·15 %.

HEKKER (The Netherlands) In our vaccine 50 % of the amount of phenol added to the vaccine can be traced back in the lyophilized pellet.

DICK (UK) What is your opinion about measurements of antibodies?

BERNSTEIN (USA) One of the factors to be considered in titrating neutralizing antibodies against vaccinia is the role of complement.

GISPEN (The Netherlands) We have used a diluent with 10 % complement in a neutralization assay in embryonated eggs. This gave a higher neutralizing antibody titre. But similar high titres were obtained when 10 % inactivated guinea-pig serum or 10 % skimmed milk was used. The increase of neutralization titre seems therefore to be an effect that cannot specifically be ascribed to complement.

BERNSTEIN (USA) When we inactivated the guinea-pig complement by heating at 60 °C for 30 min the antibody-enhancing phenomenon was destroyed. This indicates that the factor was heat-labile.

TAGAYA (Japan) I am rather sceptical about the role of guinea-pig complement in the neutralization enhancement of vaccinia virus. As already established by Briody and McCarthey, fresh guinea-pig serum has non-specific inhibitors against vaccinia virus. After addition of fresh guinea-pig serum you do not know whether or not you only measure protective neutralizing antibodies against vaccinia virus.

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CLINICAL EVALUATION OF FOUR SMALLPOX VACCINES

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Influenced by both the success of the worldwide smallpox eradication programmes and the need to develop a method of smallpox immunization which would have less morbidity associated with it, the staff of the National Institute of Allergy and Infectious Diseases (NIAID) met with an advisory group in early 1970 to discuss the problem and to propose an adequate study which would result in useful information. It was recommended that collaborative clinical trials be supported to compare the effect of dose, route and strain of vaccinia virus on the morbidity associated with smallpox vaccination and to ascertain whether or not a primary vaccination with a low dose of vaccinia virus would reduce the morbidity associated with a standard vaccination administered approximately 6 months later. The ultimate objective was to determine the best available vaccine and vaccination procedure which would give equivalent immunity to the recipient to smallpox itself and give less morbidity than that presently associated with routine vaccination in the United States. It was recognized that there would be no way of obtaining data on whether or not the vaccinee would be protected from smallpox; however, three of the investigational vaccines have been successfully used in smallpox endemic areas. Therefore, it was felt that a comparison of serologic responses might be used as indices of protection (1).

It is emphasized that this report is a preliminary presentation of data being accumulated by four individual groups of investigators participating in a collaborative programme proposed and sponsored by the Infectious Disease Branch, Collaborative Research of the NIAID. The final report and data will be published by the group elsewhere. The studies are being conducted at St Louis University; the University of California, in conjunction with the Naval Hospital, San Diego; the University of Kentucky; and the University of Colorado. The study is co-ordinated by the NIAID, and the Center for Disease Control (CDC) is serving as the reference laboratory for serologic testing.

Table I. *Titres (pfu ^{10}log) of the vaccines used in this study*

Vaccine	3	4	5	6	7	8
		(Standard 8·3)*			(Standard 8·2)	
Calf lymph	3·3	4·3	5·2	6·5	7·5	8·5
		(Standard 8·2)			(Standard 8·2)	
CAM	2·8	3·9	4·9	5·9	6·8	8·0
		(Standard 8·3)			(Standard 8·3)	
CV-1	3·1	4·1	5·1	5·9	7·0	7·6
		(Standard 8·2)			(Standard 8·3)	
Elstree	3·2	4·2	5·3	6·1	7·1	8·2

* A laboratory standard was used in each test to verify titration results.

MATERIALS AND METHODS

Vaccines

The vaccines selected for this study are:

(1) Wyeth 'Dryvax' (New York City Board of Health strain in calf lymph [calf lymph]) which is referred to as the standard vaccine in this study. In addition to being one of the study vaccines, it was used in standard concentration (10^8 pock-forming units [pfu/ml]) as the challenge vaccine 6 months following primary immunization. Two lots of licensed Dryvax were used as the challenge vaccines.

(2, 3) Two other vaccines, derived from the New York City Board of Health strain, were also used. The Lederle chorioallantoic membrane (CAM) vaccine was passaged in eggs so as to obtain a bacteriologically sterile preparation. The Rivers CV-1 strain (5) is an attenuated strain which has been used as an investigational vaccine in the United States in eczematous children. It is also grown on the CAM.

(4) The fourth vaccine was the Elstree strain, prepared in sheep lymph and provided for this study by the Lister Institute in England through the courtesy of H. G. S. Murray. Sequence of passage of this virus is rabbit/sheep/sheep. This vaccine has never been tested in the United States but has been used extensively in Europe and elsewhere throughout the world where it appears to cause less morbidity than some other vaccine strains (4).

Although the four vaccines were diluted so that the percutaneous inoculations would contain 10^8, 10^7 and 10^6 pfu/ml and the subcutaneous inoculations would contain 10^3, 10^4 and 10^5 pfu/dose, the actual initial titres are shown in Table I. Except for the 10^5 vials of the Elstree vaccine, the calf-lymph vaccine vials contain more virus in each of the doses than the other vaccines.

The four vaccines were diluted, safety tested and packaged by Wyeth Laboratories. All vaccines were bacteriologically sterile at the dilutions used for subcutaneous administration. The vaccines for subcutaneous administration were packaged in single dose tubex containers in 0·5 ml amounts. The vaccine dilutions were prepared in sterile distilled water containing not more than 1·5 mg of human serum albumin (HSA) per dose. These liquid vaccines were periodically tested and proved stable when stored frozen.

Vaccines for percutaneous administration were lyophilized following dilution in 5 % peptone. Vaccines were reconstituted at the beginning of each week by each investigator, refrigerated and discarded at the end of the week. Virus titres were stable at refrigerator temperatures for at least 3 weeks following reconstitution. The diluent for reconstituting the percutaneous vaccines was the Wyeth standard with 50 % glycerin, 0·25 % phenol and

0·005 % brilliant green, and the vaccinating needles were supplied in individual dispensing containers of 25 bifurcated needles each. Vaccine was administered by making five punctures of the bifurcated needle through a drop of vaccine.

Populations Vaccine recipients were normal children at least 1 year old. All children older than 5 years were tested serologically in order to determine whether this was a primary immunization. Any vaccinee with antibody to vaccinia virus in the pre-immunization specimen, as measured by either the haemagglutination inhibition (HI) or serum neutralization (SN) test, was excluded from the study.

Plan of study The percutaneous vaccines were prepared in identical vials and labelled with random numbers. They were distributed to each investigator so that they each received three vaccines in balance with respect to the vaccines and the three concentrations. The Elstree vaccine was delayed and was interspersed at a later date. As a result, fewer percutaneous vaccinations were given with the Elstree vaccine than with the other three vaccines.

Since there was little experience with subcutaneous inoculation of these vaccines, it was decided that each investigator would administer known concentrations and vaccines, starting with the least virus. The vaccines would then be blind-coded. Because of the positive HI serologic results with the 10^3 concentration, most of the blind study was done at this concentration and progressively less with concentrations of 10^4 and 10^5. There was initial difficulty with stability of the subcutaneous vaccines necessitating repackaging. This resulted in a delay of approximately 10 months between the majority of the percutaneous vaccinations and those administered subcutaneously.

Post-vaccination observations by the parents consisted of measurements of body temperature and daily description and measurement of the erythema and central lesion, if present, from day 0 to day 14. On day 10 the investigator saw the child, took a picture of the vaccination site, and recorded the temperature and lesion measurements. On day 28 venous blood was drawn for serologic tests. Approximately 6 months following the initial vaccination, the child was again seen, a blood specimen obtained, and challenge vaccination with calf-lymph vaccine performed. Temperature and local lesion measurements were made for 14 days. The investigator saw the child on about day 7 and took a picture of the lesion and recorded the temperature and local lesion measurements. On day 28 the postchallenge serum specimen was drawn. The parents of the vaccinee were encouraged to call the investigators if any illness occurred. In this way, non-vaccine-related fevers and other complications could be accounted for. Other data recorded included the day of maximal erythema as measured by the parent, the number of days of temperature greater than 100·9 °F falling in the 4 to 14-day period, age, sex, race of child, vaccinator, vaccine dose and route, a peak lesion score based on photographs of different smallpox reactions, and smallpox vaccination reactions as scored by the WHO. In this preliminary report, only some of the different variables and results will be analysed. The present analysis of data is based on 793 primary percutaneous vaccinations, 759 primary subcutaneous vaccinations and 946 challenge revaccinations.

Serologic testing The HI test was performed as done by the CDC and is essentially as described by Hierholzer *et al.* (**2, 3**). Chicken red blood cells were used. The SN test was that described by Wulf *et al.*(**6**) except that the MK_2-LLC cell line was used rather than primary rhesus monkey tissue cultures.

The CDC reference laboratory performed HI and SN tests on one-fourth of the serum specimens, thus giving a method of correlating the various laboratories' results with each other.

Table II. *Summary of fever data following primary vaccination among subjects with a convalescent phase HI antibody titre* $\geqslant 1:10$

Dose	Vaccine				Total
	Calf lymph	CAM	CV-1	Elstree	
			Percutaneous		
10^6	10/27 (37 %)*	7/19 (37 %)	0/9 (0 %)	6/14 (43 %)	69
10^7	12/46 (26 %)	10/37 (27 %)	5/19 (26 %)	10/30 (33 %)	132
10^8	19/63 (30 %)	17/49 (35 %)	10/36 (28 %)	16/31 (52 %)	179
Total	41/136	34/105	15/64	32/75	380
			Subcutaneous		
10^3	11/56 (20 %)	5/26 (19 %)	6/46 (13 %)	3/40 (8 %)	168
10^4	2/27 (7 %)	1/17 (6 %)	4/26 (15 %)	1/19 (5 %)	89
10^5	2/14 (14 %)	2/7 (29 %)	2/10 (20 %)	0/3 (0 %)	34
Total	15/97	8/50	12/82	4/62	291

* Number with fever > 100·9 °F/total vaccinees.

RESULTS

Vaccinees in the total study were balanced by colour and sex; however, vaccinees were not balanced by colour in each investigator's population. Seventy-nine per cent of the vaccinees were between the ages of 1 and 3 years.

Table II summarizes the fevers that occurred following primary vaccinations which resulted in a convalescent phase HI antibody titre of $\geqslant 1:10$. A positive HI titre is defined in this paper as $\geqslant 1:10$. Fever was classified as occurring if the body temperature was recorded as 101 °F or more at least once during the period 4–14 days following vaccination. Subjects with fevers of 101 °F or more recorded in the period 0–3 days following vaccination and subjects classified as having non-vaccine related complications were not included. Although there are no significant differences in fevers associated with the four vaccines administered percutaneously, 23 % who received CV-1 vaccine had a fever as compared to 43 % who received Elstree vaccine. In contrast to this, following subcutaneous vaccination only 6 % who received Elstree vaccine exhibited fevers, whereas 15 % of the vaccinees who received one of the other three vaccines exhibited fevers.

Tables III and IV show the vaccine-related complications observed in this study. These two Tables include all vaccinees whether or not they exhibited a primary take. Complications looked for prospectively included roseola vaccinatum, erythema multiforme, satellite lesions, accidental reinfection, family spread, generalized vaccinia, progressive vaccinia, eczema vaccinatum, central nervous system involvement, double primary and symptomatic local adenitis.

As indicated in Table III, data on swollen (enlarged more than 1·5 cm) or

Table III. *Vaccine-related complications following primary vaccination*

A. Percutaneous

	Calf lymph			CAM			CV-1			Elstree		
	10^6/ 74*	10^7/ 78	10^8/ 77	10^6/ 56	10^7/ 58	10^8/ 67	10^6/ 80	10^7/ 66	10^8/ 81	10^6/ 62	10^7/ 55	10^8/ 39
Roseola vaccinatum	—	—	—	1	—	2	—	—	1	1	—	—
Erythema multiforme	1	1	1	—	—	1	—	—	1	1	—	—
Satellite lesions	1	3	4	—	3	4	—	—	2	—	1	1
Accidental reinfection	—	—	—	—	—	—	—	—	—	—	—	1
Double primary	—	—	4	—	—	1	—	1	2	—	—	1
Local adenitis	—	—	—	—	—	—	—	1	1	—	—	1
Satellite lesions and generalized vaccinia	—	—	1	—	—	—	—	—	—	—	—	—
Other vaccine-related illness	—	—	2	1	—	—	—	—	1	—	—	—
Swollen arm†	—	—	—	—	—	—	—	—	—	—	—	—

Total complications/total immunized = 47/793 or 6 %.
* Total immunized.
† No data.

B. Subcutaneous

	Calf lymph			CAM			CV-1			Elstree		
	10^3/ 116*	10^4/ 60	10^5/ 23	10^3/ 64	10^4/ 48	10^5/ 20	10^3/ 125	10^4/ 94	10^5/ 26	10^3/ 90	10^4/ 71	10^5/ 22
Roseola vaccinatum	—	—	—	—	—	—	—	1	—	1	—	—
Erythema multiforme	—	—	—	—	—	—	1	—	—	—	—	—
Other vaccine-related illness	—	—	—	—	—	—	—	—	1	—	—	—
Swollen arm	11	2	—	6	4	1	6	5	—	7	3	1

Total complications/total immunized = 50/759 or 7 %.
* Total immunized.

Table IV. *Vaccine-related complications following revaccination*

A. Percutaneous followed by percutaneous vaccination

	Calf lymph			CAM			CV-1			Elstree		
	10^6/ 60*	10^7/ 60	10^8/ 59	10^6/ 42	10^7/ 40	10^8/ 50	10^6/ 63	10^7/ 45	10^8/ 56	10^6/ 39	10^7/ 41	10^8/ 33
Roseola vaccinatum	—	—	—	—	—	1	1	—	—	—	—	—
Erythema multiforme	—	—	—	—	—	—	1	—	—	—	—	—
Satellite lesions	—	—	—	1	1	—	—	—	—	1	—	—
Accidental reinfection	—	—	—	—	—	1	1	1	—	1	—	—
Family spread	1	—	—	—	—	—	—	—	—	—	—	—
Generalized vaccinia	—	1	—	—	—	—	—	1	—	—	—	—
Double primary	3	2	—	3	1	—	1	1	2	2	—	—
Local adenitis	—	—	—	—	—	—	1	—	—	2	—	—
Satellite lesions and local adenitis	—	—	—	—	—	—	—	—	—	1	—	—
Other vaccine-related illness	—	1	—	—	—	—	—	—	—	—	—	—
Satellite lesions and double primary	1	—	—	—	—	—	—	—	—	—	—	—

Total complications/total reimmunized = 34/588 or 6 %.
* Total reimmunized percutaneously with 10^8 calf lymph vaccine.

B. Subcutaneous followed by percutaneous vaccination

	Calf lymph			CAM			CV-1			Elstree		
	10^3/ 64*	10^4/ 36	10^5/ 9	10^3/ 27	10^4/ 29	10^5/ 6	10^3/ 54	10^4/ 32	10^5/ 12	10^3/ 36	10^4/ 33	10^5/ 6
Accidental reinfection	—	—	—	—	—	—	1	—	—	—	—	—
Double primary	—	—	—	—	1	—	—	—	—	1	—	—
Other vaccine-related illness	—	1	—	—	—	—	—	—	—	—	—	—
Swollen arm	—	1	—	—	—	—	—	—	—	—	—	—

Total complications/total reimmunized = 5/344 or 1·5 %.
* Total reimmunized percutaneously with 10^8 calf lymph vaccine.

Table V. *Summary of morbidity among subjects with positive HI titres* ($\geq 1:10$)

Dose	Vaccine				
	Calf lymph	CAM	CV-1	Elstree	Total
			Percutaneous		
10^6	11/27 (41 %)*	7/19 (37 %)	0/9 (0 %)	7/14 (50 %)	69
10^7	15/46 (33 %)	13/37 (35 %)	6/19 (38 %)	11/30 (36 %)	132
10^8	26/63 (41 %)	21/49 (43 %)	13/36 (36 %)	18/31 (58 %)	179
Total	52/136	41/105	19/64	36/75	380
			Subcutaneous		
10^3	12/56 (21 %)	7/26 (27 %)	8/46 (17 %)	7/40 (18 %)	168
10^4	4/27 (15 %)	2/17 (12 %)	6/26 (23 %)	2/19 (11 %)	89
10^5	2/14 (14 %)	3/7 (43 %)	2/10 (20 %)	0/3 (0 %)	34
Total	18/97	12/50	16/82	9/62	291

* Number with fever > 100·9 °F and/or vaccine-related complications/total vaccinees.

oedematous arms were not recorded following percutaneous vaccination. This parameter was not looked for until the subcutaneous vaccine administrations were begun. It is possible that some of the percutaneously vaccinated arms would have been enlarged more than 1·5 cm if the measurements had been recorded on day 0 and day 10 and compared. Only those complications that were observed are shown in each of the Tables. In general, complications following percutaneous vaccination seem to correlate with dosage, with the larger number of complications associated with the higher doses. Six per cent of the children vaccinated percutaneously and 7 % of those vaccinated subcutaneously exhibited vaccine-related complications. If the swollen-arm category is excluded in the subcutaneous vaccination complications so that a comparison can be made to the percutaneous vaccination, the percentage of complications drops to age 0·5 %.

Table IV shows that when individuals who had originally received vaccine percutaneously were revaccinated, 6 % again had vaccine-related complications. Those who received the higher doses initially had fewer reactions when revaccinated. Only 1·5 % of the individuals who were revaccinated following subcutaneous vaccination developed vaccine-related complications.

Although non-vaccine related complications were recorded and tabulated, they are not presented here because further analysis is required. They will be included in a subsequent report. The non-vaccine-related complications included mostly upper respiratory infections, otitis media, but also broken bones, etc. It was of interest to note, however, that the CV-1 vaccine was associated with more non-vaccine related complications than the other three vaccines when administered percutaneously and fewer non-vaccine related complications than the others when administered subcutaneously.

Table V summarizes the vaccine-related morbidity observed in subjects with positive HI antibody titres following percutaneous and subcutaneous primary immunization. Morbidity was classified as being present if either fever, as

Table VI. *Number of subjects with an HI titre* $\geqslant 1:10$ *following primary vaccination*

Dose	Vaccine: Calf lymph	CAM	CV-1	Elstree	Total HI's performed
			Percutaneous		
10^6	34/72 (47 %)*	20/55 (36 %)	10/79 (13 %)	20/59 (34 %)	265
10^7	55/74 (74 %)	41/51 (80 %)	24/61 (39 %)	36/54 (67 %)	240
10^8	73/76 (96 %)	64/67 (96 %)	47/69 (68 %)	36/38 (95 %)	250
Total	162/222	125/173	81/209	92/151	755
			Subcutaneous		
10^3	77/93 (83 %)	39/45 (87 %)	63/101 (62 %)	55/62 (89 %)	301
10^4	36/40 (90 %)	28/31 (90 %)	31/43 (72 %)	28/34 (82 %)	148
10^5	15/15 (100 %)	8/9 (89 %)	11/15 (73 %)	5/7 (71 %)	46
Total	128/148	75/85	105/159	88/103	495

* Number with HI titre $\geqslant 1:10$/total vaccinees.

Table VII. *Number of subjects with an SN titre* $\geqslant 1:10$ *following primary percutaneous vaccination*

Dose	Vaccine: Calf lymph	CAM	CV-1	Elstree	Total SNT's performed
10^6	18/32 (56 %)*	6/18 (33 %)	1/41 (2 %)	10/25 (40 %)	116
10^7	18/25 (72 %)	17/20 (85 %)	3/23 (13 %)	13/24 (54 %)	92
10^8	33/35 (94 %)	14/19 (73 %)	7/26 (27 %)	17/18 (94 %)	98
Total	69/92	37/57	11/90	40/67	306

* Number with SN titre $\geqslant 1:10$/total vaccinees.

defined earlier, or a vaccine-related complication, or both were present. In general, when the vaccines were administered percutaneously, there was greater morbidity at the higher dose levels. The CV-1 strain showed less vaccine-related morbidity than the other strains. Although there is variability in morbidity with the different doses of the vaccines administered subcutaneously, it does not appear to be in any consistent direction. It should be noted, however, that twice as much total morbidity followed percutaneous immunization as followed subcutaneous immunization (39 % versus 19 %).

The number of individuals who developed HI antibody titre following percutaneous and subcutaneous vaccinations is shown in Table VI. The calf lymph, CAM, and Elstree vaccines all gave 95 % or greater HI antibody seroconversion when 10^8 pfu were administered percutaneously, while the CV-1 vaccine only gave 68 % HI antibody seroconversion.

Interestingly, with subcutaneous vaccination, the 10^3 dose resulted in approximately the same percentage of HI antibody seroconversion as the 10^5 dose. In the very small numbers tested to date, only the calf-lymph vaccine has shown greater than 95% HI antibody seroconversion.

Only a small portion of the sera under study have been tested for their SN antibody titre. Table VII is presented to give the results to date. A positive serological response is defined in this paper as an SN titre $\geqslant 1:10$. Although the numbers in each group are small, the CV-1 vaccine resulted in a much lower rate of seroconversion than the other three vaccines.

Although some difficulties have been encountered with the serological tests, it has been concluded that the results from the individual laboratories are comparable. The CDC laboratory, acting as an independent laboratory with which to compare results of each investigator, has demonstrated that using twofold dilutions, 74% of all positive HI antibody tests done at the four different centres were within one tube dilution of the reference laboratory. The numbers completed to date with the SN test are too small to make any comparison, but it appears that the results obtained by the various investigators are again comparable.

DISCUSSION

As has been mentioned above, only preliminary data from this study are being reported at this time. Very little data on the revaccination response are available and only a small portion of the sera have been tested for SN titre. Further data pertaining to vaccine-related versus non-vaccine related complications should also be available at the end of the study. The data, in regard to clinical takes, has not yet been analysed.

However, with the indicated limitations, some observations can be made with the results to date. The CV-1 strain appears to be significantly different from the other three vaccines. It appears to induce less SN and HI antibody when administered either subcutaneously or percutaneously at equivalent dosage levels to the other vaccines. However, if a positive antibody response occurs, the CV-1 strain appears to cause as much morbidity as the other three strains. The calf-lymph, CAM and Elstree vaccines appear similar with respect to serological response and morbidity induced.

The majority of the fevers listed were of short duration and of low magnitude. These data will be broken down by day and height of fever. Although not shown in Table II, the total percentage of vaccinees with a subsequent positive HI antibody titre who had fever was 32% for those receiving the percutaneous vaccines and only 13% for those receiving subcutaneous vaccines.

If oedema and arm swelling are excluded as a vaccine-related complication, those receiving subcutaneous vaccine had a significantly lower rate of complications than those receiving vaccine percutaneously. Although the data have not been fully analysed, it was the opinion of the clinical investigators that there was less morbidity associated with the revaccination following primary subcutaneous immunization than following primary percutaneous immunization.

It is noted that a higher percentage of vaccine-related complications was

reported in this study than is usually expected. This may be attributable to the fact that these vaccinees were observed very carefully and parents urged to call investigation teams for any health-related problem. Therefore what might normally have been overlooked by the parent was reported in this study.

The seroconversion rate, as measured by the presence of HI antibody following subcutaneous vaccination with doses as low as 10^4 and 10^3 pfu, was impressive. The percentage of these vaccinees that seroconverted and the titres attained were similar to those noted following the standard percutaneous vaccination. It was all the more impressive when correlated with the reduced morbidity. The SN data to date corroborate these findings. However, the significance of the HI and SN titres resulting from these subcutaneous vaccines and their relationship to immunity to smallpox remains unclear.

SUMMARY

Preliminary data are presented on a prospective study to compare four smallpox vaccines (calf lymph, CAM, CV-1 and Elstree strains of vaccinia) administered at 10^6, 10^7 and 10^8 pfu percutaneously and 10^3, 10^4 and 10^5 pfu subcutaneously. These vaccinations were all followed after approximately 6 months by percutaneous vaccination with calf lymph vaccine at 10^8 pfu. The CV-1 vaccine appears to be less effective in inducing antibody formation than the other three vaccines. It appears that fewer complications are associated with subcutaneous vaccination and that fewer complications follow standard percutaneous inoculation in individuals previously given subcutaneous vaccine, although the same percentage of complications are seen in those who were previously given percutaneous vaccine.

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Discussion

LANE (USA) While the results of subcutaneous vaccination are interesting and deserve further study, the importance of a vaccination scar for large-scale campaigns in developing nations makes it imperative that we do not drop the normal intracutaneous route until very convincing definitive data are produced to show that it is not the optimal vaccination method.

GALASSO (USA) The intent of this study is not to substitute for what is currently being done by the smallpox eradication group, especially in developing nations. Actually our numbers are much too small to convince the proper authorities to make changes in standard vaccination procedures. What we are presenting is data which may show a new method of vaccination which would induce less morbidity; this would necessitate another study with much larger numbers. Our concern was not to provide a substitute vaccination procedure in developing nations but perhaps to suggest a method, once smallpox is controlled, for vaccinating individuals who travel, hospital workers or others who may need to be immunized for a variety of reasons.

EHRENGUT (West Germany) I should like to support Dr Lane concerning the importance of the development of a scar following primary vaccination. As a vaccinator I feel somehow unhappy about the terminology 'complication' as it is used in your paper. An area or an infiltration of the arm should be described just as a heavy local reaction.

GALASSO (USA) The 'complications' mentioned in the paper were indeed of a minor nature and it may be overstating the facts by referring to them as complications. However, all reactions which were not typical were referred to in this manner. As repeatedly stated, this is a very preliminary report, discussed at this time to bring this group up to date on our study; when the final report is prepared these 'complications' will be properly evaluated in terms of heavy local reaction or truly complication.

TAGAYA (Japan) In Japan comparison has been made on the clinical reactions between the CV-1 and Elstree vaccines and there were several observations different from yours. I would only like to mention here the rate of febrile reaction ($\geqslant 37{\cdot}5$ °C). Infants vaccinated by multiple pressure with CV-1 vaccine always showed a lower fever rate (less than 10 %) compared with Elstree vaccine (20–25 %).

VON MAGNUS (Denmark) What was the technique of your subcutaneous vaccination?

GALASSO (USA) The subcutaneous vaccinations were administered by Tubex vials prepared by Wyeth Laboratories. These were individual dosage vials containing 0·5 ml which were designed to deliver the stated quantity of virus. Each investigator was careful to determine that there was no drop of vaccine at the point of the needle prior to insertion. Perhaps we can call on Dr Tint of Wyeth Laboratories to elaborate on the Tubex system.

TINT (USA) The Tubex is a glass cartridge syringe which has a permanently fixed needle. It is assembled at the plant with the needle, the cartridge and the dose. It is designed with a needle that will provide a subcutaneous dose without any further handling.

NEFF (USA) One of the most severe complications in the United States is contact eczema vaccinatum. The subcutaneous administration of vaccine may in effect reduce the incidence of this complication by reducing virus spread.

GALASSO (USA) With percutaneous vaccination there is continued viral shedding for a period of days, whereas with subcutaneous vaccination there is no shedding. Aside from reduced morbidity this is an additional favourable characteristic of this vaccination procedure.

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FIELD TRIALS WITH A NEW SMALLPOX VACCINE PREPARED IN THE HUMAN DIPLOID CELLS WI-38

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There are at present three ways of producing vaccine against smallpox with regard to the substrate: the skin of calf or sheep, chorio-allantoic membrane of fertilized chicken egg, and cell culture. To improve the quality of the vaccine as to the substrate, so that the 'purity' of vaccine against smallpox can be compared with other virus vaccines, we have decided in favour of cell culture HDC WI 38.

In our country human diploid cells (HDC) WI-38 as a substrate for virus vaccine production have been placed on the same plane as primary cells(**2, 6**). Our licensing authorities have accepted live oral polio vaccine, live parenteral measles and rubella vaccines which have been registered and licensed for use on a large scale.

Experimental vaccines against smallpox from the Lister (Elstree) and Berne–Zagreb strain have been prepared on that culture. In this report we shall limit ourselves to the Berne–Zagreb strain, used by the Institute of Immunology for the production of regular batches of smallpox vaccine for the whole of Yugoslavia. We have characterized the strain on the basis of clinical reactions in 340 primarily vaccinated children with 26 different batches(**4**).

With regard to the lowest shown reactivity in preliminary field trials(**6**), we have used the Berne–Zagreb diploid vaccine for the primary vaccination of a certain number of persons above 3 years of age (legal limit for primary vaccination) during the variola epidemic in Yugoslavia in 1972.

MATERIAL AND METHODS

Tissue culture and media In all our experiments human diploid cells WI-38 were used. The number of cells in a confluent sheet ranged between 5 and 12 million in Roux bottles. The interval between the multiplication of cells and their infection, i.e. the age of cells, was 3–8 days.

Eagle's medium with 10% calf serum was used for the cell growth and medium 199 with 1% peptone (Difco) for their maintenance. Roux bottles contained 100 ml of medium. Trypsin was used in a 0·25% solution.

Seed virus Our seed virus is prepared from the Berne strain of vaccinia virus received from the Berne Institute in 1936. It is typical for the strain that since then it has been passed in Zagreb only in calf skin – hence its name Berne–Zagreb strain. From a sterile

batch of vaccine (titre 10^8 pfu/ml) the virus is purified and stabilized in HDC system by triple plaquing by the method described by Ikić *et al.* (3).

We selected virus of one plaque which through a number of passages in WI-38 cells showed the most rapid rise of titre. The material from this plaque was further passaged in HDC and the 11th passage represents seed virus of the titre of $10^{7.6}$ pfu/ml. The temperature for growth and incubation was 37 °C. Seed virus was stored at −20 °C.

Preparation of vaccine The vaccine represents the 12th passage of the Berne–Zagreb strain in the WI-38 human diploid cells.

Vaccine preparation relies on previous studies (5), which had shown that the highest virus titre was achieved on lower passage levels, up to the 27th, with younger tissues, 3 to 4-day-old cells, using medium 199 with 1 % of peptone and with the inoculum containing 1 i.u. to 1–2 cells.

We achieved the best titres when the time of incubation ranged between 48 and 72 h.

Under optimal conditions we obtained a titre of $10^{7.50}$ pfu/ml in our experiments with the Berne–Zagreb strain. This titre being unsatisfactory, we concentrated the virus using the technique of interruption of incubation at a certain stage of CPE. The bottle was stored at −20 °C. After the material had been frozen and thawed three times it was homogenized and then centrifuged at 2000 rev/min in order to remove the cell debris. The supernate without further additives represented the vaccine and was freeze-dried.

Titration and control of vaccine Titration was done by inoculating the chorio-allantoic membrane (CAM) of 12-day-old chick embryos (Leghorn breed) (1). The inoculum was 0·01 ml of virus suspension per egg. Not less than ten eggs for each of the tenfold dilutions were used for the inoculation. At least five titrations for each lot were performed. After 48 h of incubation at 37 °C the number of pocks per membrane were counted. The titre was calculated from the mean value of pock counts, the dilution and the amount of inoculum used, and expressed as pfu/ml.

The control of the substrate was performed according to the Minimum Requirements for the Selection and Use of Human Diploid Cell Strains in the Production of Virus Vaccines (Opatijá (7)) and the control of the vaccine according to WHO Requirements for Smallpox Vaccine (8).

Vaccine The batch of smallpox vaccine B-1 (Berne–Zagreb) prepared in HDC WI-38 was used for the vaccination. Its titre was $10^{7.1}$ pfu/ml and 2·5 years earlier it was $10^{7.8}$ pfu/ml.

Vaccination The preliminary primary vaccination with the Berne–Zagreb diploid vaccine was performed at the Medical Centre, Krapina, on children up to 3 years of age. In our present examination the vaccination and follow-up were carried out at the Institute of Public Health of Split, under the supervision of epidemiologists of the same Institution. A total of 724 persons were primarily vaccinated and, according to their age, were divided into the following groups: between 3 and 9 years, between 10 and 19, 20 and 39, 40 and 59, and 60 and above. All of them received only the live diploid vaccine.

Vaccination was performed on one spot on the right upper arm by the multiple pressure method (20–30 pressures). The follow-up was performed between the 3rd and 13th day and the vaccinees were asked to report to the Institute of Public Health at Split in case of stronger reactions or unforeseen complications between the daily checks or after the 13th day. Prior to vaccination, the possibility of complications was explained to all persons, and consent for vaccination was obtained from all of them (persons under age were vaccinated with the consent of their parents). All vaccinees agreed to participate in this trial, which was of particular importance for the success of the checked clinical observations. An index card was opened for each vaccinee and all relevant data entered in it.

Table I. *Reactivity of HD 11/3 vaccine in the primarily vaccinated in Split*

Rates	Age groups (years) 3–9 %	3–9 No.	10–19 %	10–19 No.	20–39 %	20–39 No.	40–59 %	40–59 No.	60 and over %	60 and over No.	%	Total no.
No fever*	85·0	51/60	72·6	127/175	74·1	275/371	64·0	64/100	27·8	5/18	72·1	522/724
Low fever†	8·3	5/60	12·0	21/175	13·7	51/371	20·0	20/100	38·9	7/18	14·4	104/724
Morbidity‡	6·7	4/60	15·4	27/175	12·1	45/371	16·0	16/100	33·3	6/18	13·5	98/724
High fever§	25·0	1/4	33·3	9/27	42·2	19/45	31·3	5/16	50·0	3/6	37·8	37/98
Prolonged fever‖	0·0	0/4	0·0	0/27	0·0	0/27	0·0	0/16	0·0	0/6	0·0	0/98

Total number of vaccinated: 724.

* No fever: temperature lower than 37 °C.

† Low fever: temperature 37–37·9 °C.

‡ Morbidity: temperature higher than 37·9 °C.

§ High fever: vaccinees from the morbidity group with temperature over 38·9 °C.

‖ Prolonged fever: vaccinees from the morbidity group with temperature lasting over 2 days.

Clinical reactions Clinical reactions of those vaccinees who after primary vaccination showed a typical primary vaccinia after 7 days (the day following vaccination was counted as the first day) were followed up.

Among clinical reactions particular attention was paid to the increase and duration of fever. The temperature was taken axillary twice a day, beginning from the fourth day after vaccination. No vaccinee was given antipyretics.

With regard to the fever, the successfully vaccinated children were divided into three groups. The first group, marked 'no fever', covered persons whose temperature was lower then 37 °C. Vaccinees having a temperature of from 37 to 37·9 °C were put in the second group, marked 'low fever'. The third group consisted of persons whose temperature was higher than 37·9 °C. This group was marked as 'morbidity' group. The rates were always expressed in percentages. Within the 'morbidity' group we had two subgroups in which the 'high fever' one represented temperature higher than 38·9 °C. The rate was expressed as the percentage of vaccinees with this high temperature from the number of vaccinees in the 'morbidity' group. The subgroup 'prolonged fever' represented the duration of temperature for two days or longer. The rate was expressed as the percentage of persons with the prolonged duration of temperature from the number of persons covered by the 'morbidity' group.

RESULTS

At the moment of outbreak of the epidemic we had at our disposal only one tested batch, the B-1 diploid vaccine of the Berne–Zagreb strain titre $10^{7\cdot1}$ pfu/ml.

In the field trial we kept evidence of the take and reactivity of the vaccine, following the primarily vaccinated persons. The percentage successfully vaccinated was 100, which is much more than in our earlier trials. Local reactions were very mild, with small erythema and mild swelling. Of the clinical symptoms, our attention was mainly concentrated upon the height and duration of temperature.

Table I shows the temperature reactions in the vaccinees at Split, divided by age groups, and total. Out of a total of 724 vaccinees, 522 or 72·1 % had no fever, 104 or 14·4 % belonged to the 'low fever' group, while 98 or 13·5 % belonged to the 'morbidity' group. Of these, 37 persons or 37·8 % belonged to the 'high fever' group, but none of them had a high temperature for 2 days or longer, i.e. the 'prolonged fever' group was negative. There have been no cases of post-vaccinal complications. There is a striking and significant difference in the 'no fever rate' and 'morbidity rate' between the first and the last age group among these vaccinees.

If we separate the results of the first age group, i.e. that between 3 and 9 years, and compare it with our earlier results of the reactivity of the diploid vaccine in vaccinated children up to 3 years of age at Krapina (Table II), we shall see that the results tally with the exception of the 'high fever rate', which is higher in the group of older children than in the group up to 3 years of age. We must, however, point out that these 25 % in fact represent one child with high fever out of a total of four in the 'morbidity' group, which is certainly negligible. Not one child had a temperature above 38·9 °C in the Krapina field trial.

If we compare the values from Table II with the results of reactivity in pri-

Table II. *Comparison of reactivity of HD 11/3 in the primarily vaccinated in two field trials*

Rates	Field trial Krapina* children under 3 years		Field trial Split† children 3–9 years	
	%	No.	%	No.
No fever	78·6	33/42	85·0	51/60
Low fever	9·5	4/42	8·3	5/60
Morbidity	11·9	5/42	6·7	4/60
High fever	0·0	0/5	25·0	1/4
Prolonged fever	0·0	0/5	0·0	0/4

* Vaccine lot B-1 (titre $10^{7\cdot6}$ pfu/ml).
† Vaccine lot B-1 (now titre $10^{7\cdot1}$ pfu/ml).

marily vaccinated children up to 3 years of age with the Berne–Zagreb calf-lymph vaccine, we shall see great differences (Table IV). Under 'A' the group of examinees was vaccinated with calf-lymph vaccine batches of higher titres ($10^{8\cdot0}$ to $10^{8\cdot45}$ pfu/ml), and under 'B' with vaccine batches of lower titres ($10^{6\cdot8}$ to $10^{7\cdot4}$ pfu/ml). As proved earlier(4), vaccine titre did not influence the reactivity. The difference between the diploid and the calf-lymph vaccine, with regard to reactivity, is shown in the 'no fever', 'low fever' and, most important, 'morbidity' rate. The reactivity of the calf-lymph vaccine is obviously much higher compared to the diploid vaccine.

If we return to Table I, we note that the 'no fever rate' drops slowly, going from the lower to the higher age groups, and is significantly smaller than the

Table III. *Comparison of reactivity of HD 11/3 in the primarily vaccinated persons of 60 years and older and of CL* vaccine in the primarily vaccinated children*

Rates	Percentage		
	HD 11/3 vaccine, 60 years and older	CL Berne–Zagreb vaccine Children up to 3 years	
		A	B
No fever	27·8	11·8	27·1
Low fever	38·9	54·4	42·1
Morbidity	33·3	33·8	30·8
High fever	50·0	8·7	12·1
Prolonged fever	0·0	17·4	15·2

* CL = Calf lymph.

Table IV. *Comparison of reactivity of HD 11/3 and CL* vaccines in primarily vaccinated children*

Rates	Field trial Krapina,† children up to 3 years HD vaccine		Field trial Split,‡ children 3–9 years HD vaccine		Calf lymph Berne–Zagreb§ Children up to 3 years			
					A		B	
	%	No.	%	No.	%	No.	%	No.
No fever	78·6	33/42	85·0	51/60	11·8	8/68	27·1	29/107
Low fever	9·5	4/42	8·3	5/60	54·4	37/68	42·1	45/107
Morbidity	11·9	5/42	6·7	4/60	33·8	23/68	30·8	33/107
High fever	0·0	0/5	25·0	1/4	8·7	2/23	12·1	4/33
Prolonged fever	0·0	0/5	0·0	0/4	17·4	4/23	15·2	5/33

* CL = Calf lymph.

† Vaccine lot B-1 (titre $10^{7\cdot6}$ pfu/ml).

‡ Vaccine lot B-1 (now titre $10^{7\cdot1}$ pfu/ml).

§ A: 6 vaccine lots (CL) with titres ranging from 10^{8} to $10^{8\cdot45}$ pfu/ml. B: 8 vaccine lots (CL) with titres ranging from $10^{6\cdot8}$ to $10^{7\cdot4}$ pfu/ml.

others in the last group of examinees (60 years and above). This drop is at the expense of the 'morbidity rate', which in this last age group is much higher. From this we could conclude that the reactivity of the vaccine increases with the age of the vaccinees. It is, however, mild on the whole and corresponds to the reactivity of the calf-lymph vaccine children under 3 years of age (Table III).

DISCUSSION AND CONCLUSION

Special circumstances in Yugoslavia this year enabled primary vaccination against smallpox of persons belonging to different age groups, including those of 60 years and over. In this field trial we confirmed our preliminary results, which pointed to a far lower reactivity of the diploid smallpox vaccine as compared to the Berne–Zagreb calf-lymph vaccine. These differences appear evident from fever rates, especially morbidity rate, even in the age group comprising children of 3–9 years. A small percentage of persons had high fever while prolonged fever was not recorded in any of the persons who had received the diploid vaccine. It was observed, though, that the reactivity of the diploid smallpox vaccine increased with the age of vaccinees, but even in the age group of 60 years and over it just reached the reactivity produced in children under 3 years old by the calf-lymph vaccine.

A 100% successful vaccination rate and also a very satisfactory serological response, the results of which have not been presented in this paper, confirm good antigenicity of the diploid smallpox vaccine.

This leads us to the conclusion that after 11 passages on the suitable substrate of human diploid cells WI-38, including 3 plaque passages, we obtained a further attenuated vaccinal strain.

SUMMARY

In the course of a smallpox epidemic in Yugoslavia in 1972 it became evident that a certain percentage of adults had not been primarily vaccinated. In view of previous experience on the low reactivity of the HDC WI-38-grown smallpox vaccine, Berne–Zagreb strain, we used this vaccine for the primary vaccination of 724 persons from 3 to over 60 years of age. The successful vaccination rate was 100%.

Although the reactivity of the HD vaccine, with regard to fever indices, increased with the age of vaccinees, it was far below that in those vaccinated with the calf-lymph smallpox vaccine. This shows that the strain had become further attenuated after the plaquing and the passage in the WI-38 human diploid cells.

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Discussion

NETTER (France) What was the proof that your vaccine at the 12th passage was still protective against smallpox during the last epidemic in Yugoslavia?

WEISZ-MALEČEK (Yugoslavia) We reimmunized one physician who was in charge of a smallpox hospital and he is still living. We have only this case.

HOCHSTEIN–MINTZEL (West Germany) Table I shows that primary vaccinations were done up to the age of 60 and over. Are there any indications of complications as to the age at primary vaccination?

WEISZ–MALEČEK (Yugoslavia) There are no data available. Vaccines from other producers with different strains were used.

LANE (USA) The Yugoslavs did an excellent job under very difficult conditions. In the face of smallpox no special precautions or techniques should be used. *Everyone* should be vaccinated. Thus the lack of data on the means, strains, precautions and contra-indications in the recent outbreak is quite understandable.

WEISZ–MALEČEK (Yugoslavia) In the whole of Yugoslavia about 18000000 doses of smallpox vaccine were administered during the epidemic of 1972 for primary and revaccinations. About 6500000 doses were our own production (Bernic–Zagreb, calf vaccine). The others were different vaccines (different strains) as a gift from WHO and various countries. The dispersion of different vaccines was not analysed. It will be done in the near future.

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FIELD WORK WITH A STABLE FREEZE-DRIED SMALLPOX VACCINE PREPARED IN MONOLAYERS OF PRIMARY RABBIT KIDNEY CELLS

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Methods now generally used for virus production and testing enable us to produce live virus vaccines which meet consistently high standards of safety concerning all kinds of extraneous micro-organisms including viruses. Although the introduction of the seed virus principle, the use of a more defined strain of vaccinia virus and in many places the use of freeze-dried vaccine instead of liquid glycerinated vaccine improved the safety and stability, the more recent developments in virology are not yet introduced in the production and testing of smallpox vaccine.

The most common source of vaccinia virus for the production of smallpox vaccine is still the skin of living animals. A smaller or wider area of the animal skin is scarified and vaccinia virus is rubbed into the scarifications. Four or five days later the animal is sacrificed by exsanguination and the vaccinal eruptions on the skin are collected with a sharp spoon. Although aseptic conditions enable us to keep the number of bacteria in the pulp very low, the use of 0·5 % of phenol for a further reduction of this number is inevitable.

Vaccines prepared from this pulp have been used for vaccination of humans against smallpox for many decades and have been shown to be immunogenic as well as protective against smallpox.

A second source of vaccinia virus for the production of smallpox vaccine is the chorioallantoic membrane (CAM) of chicken eggs or cell cultures derived from chick

Table I. *Conditions critical for acceptance of a new smallpox vaccine*

Work	Study	Define
Laboratory	Qualities of the vaccine	Potency stability (including extraneous viruses), strain, seed virus system, number of passages removed from calf skin
Field	1. Primary vaccination of adults	Take rate, vaccination illness, antibody production
	2. Revaccination of adults	Take rate, local reaction, rise of antibody titre
	3. Revaccination of adults with the new vaccine and subsequently the use of the new and the conventional vaccine	Take rate, local reaction, rise of antibody titre
	4. Large-scale application of the new vaccine	Major and minor local and general side effects
	5. Double-blind field trial in smallpox endemic area	Possibly not feasible

embryos. Although such smallpox vaccine is free from bacterial contamination the presence of the fowl leucosis complex of viruses cannot be concluded. The question whether or not these viruses are pathogenic for man is not relevant: a vaccine should only contain the agent indicated on the label.

In this paper the production of freeze-dried smallpox vaccine made in monolayers of primary rabbit kidney cells and the field work which is done with this vaccine is described. Primary rabbit kidney cells were chosen for the same reason as their use in rubella vaccine production because despite the use of all available testing methods no adventitious agents could be isolated from monolayers of these cells.

The seed virus used for the production of the tissue culture smallpox vaccine was the Elstree strain of vaccinia virus obtained from the Lister Institute of Preventive Medicine, England. It was the same sterile seed as used for the production of calf lymph so that the vaccinia virus in the vaccine was not more than one passage removed from the calf to which it was adapted. When introducing a new smallpox vaccine for mass vaccination it is necessary to make sure in a number of experiments that the vaccine is at least as effective as the conventional vaccine. The conditions critical for acceptance of such a vaccine are given in Table I.

The experiments mentioned in this Table must be done before a new smallpox vaccine can be accepted for routine use. A double blind field trial in a smallpox endemic area is possibly no longer feasible.

Table II. *Titre (log pfu/ml) of tissue-culture smallpox vaccine and calf lymph after storage for several weeks at −4 °C and +37 °C.*

(Both types of vaccine were freeze-dried in 0·20 ml amounts with 5% sorbitol and sealed *in vacuo*.)

Storage (weeks)	Titre			
	Tissue culture vaccine		Calf lymph	
	−4 °C	+37 °C	−4 °C	+37 °C
0	8·2	—	8·4	—
4	8·3	8·1	8·5	8·1
8	8·4	8·2	8·5	8·1
12	—	8·2	8·2	7·6
17	—	8·0	8·4	7·9
23	—	7·7	—	—
27	8·4	7·7	8·3	7·3

PRODUCTION AND TESTING OF THE VACCINE

The production, safety- and potency-testing of the vaccine will be published *in extenso* elsewhere(**1**, **2**). For this reason it should be sufficient to describe briefly that monolayers from rabbit kidneys were prepared using standard procedures. The vaccinia virus harvested from inoculated monolayers was concentrated ten times by ultrafiltration lyophilization in 0·2 ml amounts was done in McIlvaine's buffer containing 5% peptone and 5% sorbitol. The ampoules were sealed *in vacuo*.

Potency tests were done using the pock-counting technique on the chorioallantoic membrane of 12-day-old chick embryos. A freeze-dried reference vaccine was titrated daily. The WHO. International Reference for smallpox vaccine was titrated twice a year. The ^{10}log titres of this vaccine were found to be between 8·4 and 8·6 pock-forming units per ml. The stability test is a titration as described of the vaccine after 4 or more weeks of incubation at 37 °C(**4**).

Table II shows the stability of tissue culture smallpox vaccine and calf lymph during 27 weeks of storage at −4 °C and +37 °C. Both types of vaccine were freeze-dried in 0·20 ml amounts with 5% peptone and 5% sorbitol.

The data show that during storage at 37 °C the stability of the tissue culture smallpox vaccine is higher than that of the calf lymph. The loss of virus after 27 weeks is about 0·5 and 1·0 log respectively for the tissue culture vaccine and the calf lymph.

Needless to say that after extensive testing the cells used for production of the vaccine were always found to be free of extraneous agents.

FIELD WORK

PRIMARY VACCINATION OF ADULTS

In order to study the local reaction and the disease-inducing capacity of the tissue culture vaccine, 120 adults were vaccinated primarily against smallpox with tissue culture vaccine and calf lymph alternatively. Fifty-nine people were vaccinated with the tissue-culture vaccine and 61 with the calf lymph. The age of the vaccinees varied between 6 and 49 years and was evenly distributed among both groups. The vaccinees were requested to take their body temperature daily at 19.00 h in a period lasting from the first day after the vaccination till the 13th day. The vaccinees were also requested to come to the office 3 weeks after the vaccination. At this visit they were questioned about headache, neck pain and axillary pain during this 3-week period.

All vaccinations lead to a typical primary vaccinia. In Table III A the number of vaccinees is given showing a body temperature of $\geqslant 38{\cdot}5$ °C (101·3 °F) on at least 1 day during four consecutive periods of 3 days each. For this purpose the period of 13 days after the vaccination was divided into four periods of 3 days each. The data show that where fever is concerned there is no significant difference between the two vaccines applied.

From Table III B it may be concluded that there is again no difference between the two vaccines with respect to headache, neck pain or axillary pain during a period of 3 weeks after vaccination.

Serologic investigation in this group of vaccinees was impractical; however, we were able to take a blood sample from some of them 1 year later. Of the group vaccinated with calf lymph, 25 blood samples became available, and of the cell culture vaccine group, 15. The serum titrations were done against vaccinia virus strain Elstree with a plaque reduction technique using monolayer cultures of continuous rabbit kidney cell line RK 13 in Seighton tubes. Twofold dilutions were tested, per dilution four tubes were used. The antibody titres were calculated according to Reed and Muench. The mean antibody titres ($\log^2$) of the two groups is given in Table III C.

The data show that the antibody titres 1 year after vaccination with calf lymph or with tissue culture smallpox vaccine do not differ from each other. This type of investigation will be repeated in a larger group of adults, taking pre-serum samples before vaccination and post-serum samples 8 weeks after vaccination.

PRIMARY VACCINATION OF INFANTS

Infants between 6 and 12 months old were vaccinated alternatively with calf lymph or with tissue culture vaccine. In this way 58 infants were vaccinated with calf lymph and 59 with tissue culture vaccine. The mothers were requested to come back with their vaccinated child 14 days after vaccination, when they were questioned about vaccination illness of the infants. The result is given in Table IV A. It should be noted that in two children the vaccination with tissue culture vaccine was not successful. On the other hand one of the children successfully vaccinated with tissue culture vaccine had been vaccinated with calf lymph twice before. There appears to be no difference in frequency of vaccination illness after primary vaccination of infants with these two vaccines.

Table III. *Primary vaccination of adults*

III A. *Number of vaccinees with fever* ⩾ 38·5 °C (101·3 °F) *on at least* 1 *day during four consecutive periods of* 3 *days each after primary vaccination of* 120 *adults with calf lymph* (61) *or tissue culture smallpox vaccine* (59)

Days after vaccination	Vaccinees with fever ⩾ 38·5 °C (101·3 °F) out of a total of 120 vaccinated with	
	Calf lymph (61)	Tissue culture vaccine (59)
1–3	2	0
4–6	0	1
7–9	11	8
10–12	8	6

III B. *Headache, neck pain and axillary pain in a period of* 3 *weeks after primary vaccination against smallpox of* 120 *adults with calf lymph* (61) *or with tissue culture vaccine* (59)

Complaints	Vaccinees with complaints out of a total of 120 adults primarily vaccinated with	
	Calf lymph (61)	Tissue culture vaccine (59)
Headache	35	26
Neck pain	16	12
Axillary pain	44	40

III C. *Mean antibody titre* (log^2) *in adults one year after primary vaccination with calf lymph or with tissue culture smallpox vaccine*

	Number of sera	Mean antibody titre (log^2)
Calf lymph	25	6·6
TC vaccine	15	6·9

The infant's weight was determined before and 14 days after vaccination. The children were split up into three categories according to the changes in weight before and after vaccination. The results are given in Table IV B. There is also no difference in changes of weight after vaccination with these two vaccines. Of the seven children vaccinated with calf lymph and the nine children vaccinated with tissue culture vaccine who did not show a weight increase or had a slight

Table IV. *Primary vaccination of infants*

IV A. *Vaccination illness as noticed by the mother of 6 to 12-month-old infants during a period of 14 days after primary smallpox vaccination with calf lymph (58 infants) and tissue culture vaccine (59 infants) alternatively*

Vaccination illness	Vaccine	
	Calf lymph	Tissue-culture vaccine
No vaccination scar	0	2
No illness noticed	25	24
Slight local reaction	21	27
Moderate local and general reaction	12	6
Total	58	59

IV B. *Changes in weight after primary smallpox vaccination of infants with calf lymph (58 infants) and tissue culture vaccine (59 infants) alternatively*

Changes in weight	Vaccine	
	Calf lymph	Tissue-culture vaccine
No increase or slight loss	7	9
Increase $\leqslant$ 150 g	17	15
Increase $\geqslant$ 150 g	27	30
Not determined	7	5*
Total	58	59

* These 5 include the two children without vaccination scar.

IV C. *Vaccination illness as noticed by the mothers of 6- to 12-month-old children who did not show a weight increase or had a slight loss of weight after smallpox vaccination with calf lymph or tissue culture vaccine*

Vaccination illness	Vaccine	
	Calf lymph	Tissue-culture vaccine
No illness noticed	2	3
Slight local reaction	2	5
Moderate local and general reaction	3	1
Total	7	9

Table V. *Routine vaccination of* 6- *to* 12*-month-old infants with tissue culture smallpox vaccine, take-rate, number and percentage of children with satellite pocks or vaccination illness as noticed by the mother during a period of* 14 *days after the vaccination*

Infants	Number	Percentage
Vaccinated	473	100
Take-rate	448	94·7
Satellite pocks	21	4·7
Vaccination illness	64	14·3

loss, the vaccination illness as noticed by the mother is given in Table IVc. The data in this Table suggest that a slight loss of weight of infants after smallpox vaccination had no bearing on the severeness of the vaccine illness.

From this study it can be concluded that although the number of children is small, no difference in frequency and severity of vaccination illness and growth rate of infants vaccinated with calf-lymph or with tissue-culture vaccine was observed.

ROUTINE APPLICATION OF TISSUE CULTURE VACCINE IN INFANTS

The tissue culture smallpox vaccine was used in the routine vaccination of 6- to 12-month-old infants. A total of 473 children were vaccinated by three vaccinators within a period of approximately 6 months. The take rate, number and percentage of children with satellite pocks or vaccination illness as noticed by the mother during a period of 14 days after the vaccination is given in Table V. No differences have been noted between children vaccinated with tissue culture smallpox vaccine and children vaccinated with calf lymph prepared from the same strain and reported previously by Polak(3).

REVACCINATION OF ADULTS

Co-workers of the R.I.V. who had been vaccinated with calf lymph 3–6 years previously were revaccinated alternatively with calf lymph and tissue culture vaccine.

Blood samples were drawn before and 2 months after revaccination. The pock was inspected four days after revaccination. If a vesicle or a rest of a vesicle was visible the revaccination was considered to have been successful. Using this criterion all 89 revaccinations except one were successful.

The sera from 24 revaccinees were titrated in the plaque reduction technique as described before. The sera from the other 65 revaccinees were titrated with the immunofluorescent technique in the Virus Diagnostic Department of the R.I.V.

The mean fold increase in antibody titre for both methods is given in Table VI. From these results it can be concluded that the rise in antibody titre after revaccination of adults against smallpox with calf lymph or with tissue culture vaccine

Table VI. *Mean fold increase in antibody titre before and 2 months after revaccination of adults with calf lymph and tissue culture vaccine alternatively. The antibody titrations were done partly with the plaque reduction technique and partly with the immunofluorescence technique*

	Calf lymph		Tissue culture vaccine	
Technique	Number of revaccinees	Mean fold increase in antibody titre	Number of revaccinees	Mean fold increase in antibody titre
Plaque reduction	11	2·7*	13	2·5†
Immunofluorescence	32	2·0	33	1·9

* One revaccinee showed a slight decrease in antibody titre.
† Two revaccinees showed a slight decrease in antibody titre.

is of the same order of magnitude both in plaque reduction and in the immunofluorescent technique. This revaccination of co-workers will be continued. Three years later the revaccinees will be revaccinated again with the tissue culture vaccine and with the calf lymph.

Large-scale application in a tropical area of the tissue culture vaccine is in preparation. A double-blind field trial in a smallpox endemic area is probably neither feasible nor necessary for acceptance of this vaccine for routine application against smallpox in the Netherlands.

SUMMARY

A stable freeze-dried smallpox vaccine prepared in monolayers of primary rabbit kidney cells and free of extraneous agents was compared in the field with freeze-dried calf lymph from routine production. The vaccines were used for primary vaccination of 120 adults and 590 infants and for revaccination of 89 adults. Between both vaccines no differences were observed in relation to take-rate, antibody-inducing capacity and the development of vaccination illness, under which fever, local and slight or moderate general reactions.

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Discussion

APPLEYARD (U.K.) I would like to make a general comment on the titration of antibodies against vaccinia virus. I feel that when we are considering the relative value of different methods we should keep in mind what we know of the factors that are likely to be important in immunity to smallpox. Now these include in general terms cell-mediated mechanisms and antibody. It is probable that both are essential to have full immunity. Now cell-mediated mechanisms are probably very important but there is no simple method of estimating these. So I think in the present discussion we can ignore them, unfortunately. It is well known that there are many different antibodies produced in response to smallpox infection; at least 20 have been detected by immunodiffusion. The only ones that are likely to be implicated in immunity are neutralizing antibodies against extracellular virus. The conventional neutralizing antibody tests and haemagglutination inhibiting antibodies are directed against intracellular virus. Now I think at the moment we cannot make any definite statement on the role of these three antibodies but we have evidence, some of which I presented earlier, that the most important antibody is the antibody directed against extracellular virus, and if anyone wants, I could give further evidence for this. My feeling is that at the moment neutralizing antibody measured in the conventional way and haemagglutinating inhibiting antibody are possibly concomitants of immunity but they themselves are not directly important. So what I would like to say is that surely it is worth while to put in the extra effort involved to estimate the amount of antibody against extracellular virus.

LANE (USA) I would like to point out an essential purely statistical problem. If we do mean geometric mean antibody titres rather than the percentage of seroconversion of primaries, in most laboratories we generally get into a situation where there is a cut-off dilution. In fact, we are not really measuring the true biological spectrum of antibody titres, we are measuring our truncated estimate of that, and statistically this may have very little meaning when we do not try to make these into a mean figure and sum up the aggregate of a group of people whose sera are titrated.

DEKKING (The Netherlands) What is the advantage of using this relatively unknown system of primary rabbit kidney cells above, for instance, the chorioallantoic membrane of chick embryos? I would be surprised if primary rabbit kidney cells were free of viruses. In eggs we know that they exist and we have methods to detect them.

HEKKER (The Netherlands) Many rabbits are tested with all available methods and no viruses are found.

EHRENGUT (West Germany) I must support Hekker. We know also from the studies in Belgium that the primary rabbit kidney cells can be used for vaccine production. A large-scale study was done without finding any extraneous virus.

BENENSON (USA) Why do we not use a continuous cell line like RK_{13} cells for vaccine production?

HEKKER (The Netherlands) This is the general problem of the acceptance of continuous cells for vaccine production. I think we shall have to organize another conference to cover that.

NETTER (France) With RK_{13} cells injected into cortisone-treated hamsters one can get the same percentage of tumours as with HeLa cells.

TINT (USA) There are a number of presumably diploid rabbit cell lines under study. Based on karyology, these look promising in contrast to primary cultures, which may have rather bizarre karyology. There are at least 100 passages and the cells are still diploid.

SESSION V

COMPLICATION FOLLOWING VACCINATION

Chairman: Dr J. M. LANE (USA)
Secretary: Dr Ch. A. HANNIK (The Netherlands)

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DECREASE OF POSTVACCINAL DEATHS IN AUSTRIA AFTER INTRODUCING A LESS PATHOGENIC VIRUS STRAIN

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Until a few years ago, Austria was among the countries where neurological complications following smallpox vaccination were rather common. From 1947 to 1951, 43 deaths after postvaccinal encephalitis were recorded from a total of 398595 primary vaccinations (Table I). In all these cases, neurohistological examination revealed the characteristic picture of diffuse (disseminated) perivenous focal encephalitis of Spatz-type, or microglia encephalitis according to de Vries. A sharp drop in postvaccination mortality from encephalitis was observed after the age limit for primary vaccination was set (from 1951 onwards) to the end of the third year of life, and in the period after 1965 fatal encephalitic complications could be completely eliminated by restricting primary vaccination between the age of 2 and 3 years.

In Austria all cases of death following smallpox vaccination, even with no apparent causal connexion with the vaccination, have to be reported to the public health services. In addition, in each case of postvaccinal death a post-mortem has to be done. Therefore we are able to obtain accurate and complete data of fatal non-encephalitic postvaccinal complications, including those with no neurohistological sign of perivenous focal encephalitis but with brain oedema or no pathological change in the central nervous system.

In Austria previously deaths from non-encephalitic complications were more frequently observed among infants and young children than deaths from postvaccinal encephalitis; however, since in nearly all cases other potential causes such as enterocolitis, pneumonia, bronchiolitis, purulent meningitis, otitis media, etc., were incriminated, these accidents were not brought into causal, but rather casual connexion with vaccination. The important role which fatal non-encephalitic complications may have in a smallpox vaccination programme is clearly shown by Austrian public health statistics covering the period from 1959 to 1970. In this period, in addition to 11 fatal cases of postvaccinal encephalitis, as many as 62 deaths from non-encephalitic complications were recorded within 3 weeks of primary smallpox vaccination (Table II).

There are several points which suggest that smallpox vaccination is a contributing factor in the majority of fatal non-encephalitic complications. This view is supported by the observation that fatal complications of this type occur generally within a closely limited period of time after vaccination, i.e. when vaccination

Table I. *Number of deaths due to postvaccinal encephalitis in Austria* (1947–1970)

	Primary vaccination (successful)	No. of encephalitic deaths	One case of encephalitic death/no. of primary vaccinations	No. of cases of encephalitic death/100000 primary vaccinations
1947–51	398·595	43	9·269	10·8
1952–56	349·648	15	23·309	4·3
1957–61	421·864	18	23·436	4·3
1962–66	530·453	8	66·306	1·5
1967–70	412·599	0	—	0·0

Table II. *Postvaccinal cases of death in Austria* (1959–70)

	Cases of death due to postvaccinal encephalitis	Non-encephalitic cases of death	Total of postvaccinal deaths	Primary vaccination (successful)
1959	2	7	9	86·543
1960	—	9	9	91·814
1961	1	4	5	77·762
1962	1	7	8	130·622
1963	5	7	12	107·520
1964	2	8	10	88·012
1965	—	6	6	106·335
1966	—	7	7	97·964
1967	—	2	2	105·762
1968	—	3	3	105·256
1969	—	1	1	100·732
1970	—	1	1	100·849

sickness tends to reach, and is passing, its climax (from the middle of the first week to the end of the second week after vaccination). Prior to and after this period fatal complications of this type are less common. Otherwise the coincidence of a high death-rate and vaccination would be accidental.

As mentioned above, in practically all fatal cases of non-encephalitic aetiology post-mortem examination reveals that vaccinal infection is associated with concomitant infectious conditions such as pneumonia, bronchiolitis, enterocolitis, otitis media, and others. Death is likely to be due to the fact that the child's organism cannot cope with the stress of two infections and fails to recuperate.

The various adverse interactions between vaccination sickness and other concomitant infections (e.g. synergism of infections, synergism of toxins) have been described recently by Stickl & Helming (**11**), Hoedt & Pfeifer (**3**), Mahnke (**6**) and others. It is our suggestion

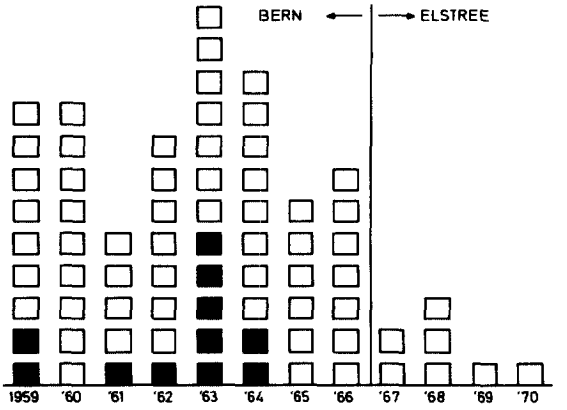

Fig. 1. Postvaccinal fatalities in Austria after primary smallpox vaccination with virus-strain Bern (1959–66) and virus strain Elstree (1967–70). ■, Cases of death caused by postvaccinal encephalitis or encephalopathies. □, Non-encephalitis cases of death.

that an outburst of a concomitant infection after vaccination may be due to the activation of latent infectious processes by vaccination. As the clinical manifestations of concomitant infections are masked by vaccination sickness, they may not be noticed, or are diagnosed too late, therefore this component of the double infection is practically not preventable. It seems that the only means of ensuring an efficient decrease in the intensity of all stress factors involved is an appropriate modification of vaccination – for instance, the use of a vaccine which has been prepared from a vaccinia virus strain of not too high reactogenicity. This necessitates the use of a vaccinia virus strain which does not produce marked systematic disturbance that might seriously interfere with the functions of the body. The demand for vaccinia virus strains of low reactogenicity has been frequently expressed by various authors (**1**, **4**, **5**, **7**, **9**, **10** *et al.*).

In Austria the vaccinia virus strain 'Bern' was used in the preparation of smallpox vaccine up to 1966. Since 1967 smallpox vaccinations have been made with vaccinia virus strain 'Elstree'. We selected the strain Elstree considering the investigations of Polak *et al.*(**8**), who showed that strain Elstree is of much lower pathogenicity than is strain Bern. As demonstrated by Table II and Fig. 1, the incidence of fatal postvaccinal non-encephalitic complications decreased from 6·8 cases per year (for the years 1959 to 1966) to 1·8 per year (for the years 1969–1970), in consequence of the introduction of the strain Elstree. This divergence is statistically highly significant as proved by the Institute of Medical Statistics and Documentation in Vienna.

The abrupt drop in the incidence of fatal non-encephalitic cases by approximately 75 % after introduction of a safer vaccine clearly demonstrates that in the majority of these cases death was in causal connexion with vaccination. On the other hand, in most of the few cases of fatal postvaccinal complications which still occur, an accidental coincidence with vaccination is highly probable in accordance with the views of Ehrengut & Ehrengut-Lange(**2**), inasmuch as the low rate of fatal cases occurring after smallpox vaccination is not essentially greater than the fatal cases following other vaccinations, e.g. poliomyelitis vaccination, where undoubtedly the accidental character is evident.

SUMMARY

As the result of various measures taken by the public health service in the last twenty years, the number of deaths after smallpox vaccination in Austria decreased to a small proportion of the death losses recorded previously. The deaths caused by postvaccinal encephalitis practically disappeared after primovaccination had been restricted to the second and third year of life. Non-encephalitic, postvaccinal cases of death of which the causal connexion with the vaccination was not given due attention in former times was reduced to nearly 25 % of those observed previously by omitting vaccination in the first year of life and introducing a less pathogenic virus-strain (Elstree) for vaccination. This is why today deaths following smallpox vaccination are hardly more frequent than those caused by other vaccinations.

We thank Prof. Wohlzogen (Institute for Medical Statistics and Documentation) and Dr Lorant (Board of Public Health, Vienna) for advice and help in statistical matters.

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Discussion

S. S. MARENNIKOVA (USSR) Have there been other changes in vaccination policy besides the introduction of the Elstree strain in 1967?

W. HEINRICH (Austria) We are not aware of any other change.

S. S. MARENNIKOVA (USSR) Is the vaccination age still the same?

W. HEINRICH (Austria) No, we prefer to vaccinate only children of 2 and 3 years.

J. M. NEFF (USA) Do you have a slide or a table with characterization of the non-encephalitic deaths and the age at which they occur?

W. HEINRICH (Austria) No, I have not.

W. EHRENGUT (West Germany) The theme of Dr Heinrich's paper has been subject to some controversial opinions between Dr Berger from Austria and ourselves. Considering deaths due to various causes like pneumonia, for instance, following mass vaccinations, many factors have to be taken into account, e.g. intercurrent epidemics of virus infections such as influenza, Echo and Coxsackie-infections. We think that the vaccination itself, no matter whether it is a polio-vaccination with Sabin vaccine or the Jennerian vaccination, has nothing to do with these complications (W. Ehrengut & J. Ehrengut-Lange, 1969, Interkurrente tödliche Erkrankungen nach Polioschluckimpfung und Pockenschutzimpfung, *Münch. med. Wschr.* **111**, 1092–1099). Dr Heinrich's statement that he believes death to be purely coincidental satisfies me. The decrease in non-encephalitic deaths in Austria started as early as 1965–6, when the Bern strain was still in use. Therefore there must be other causes – for example, the age of the vaccinees, which has been changed from the first year of life into the second.

H. STICKL (West Germany) We had a rather important number of cases of encephalitis after vaccination in Bavaria in 1958, which declined spontaneously. Because of interference with other infectious diseases a trial was set up with two vaccines, one prepared from the Bern strain, the other from the Elstree strain. During the first year all primary vaccinations in one half of the country were performed with one vaccine, in the other half with the other vaccine; during the second year the vaccines were changed. A clear falling off in the number of encephalitis as well as of other complications was observed. Though the strain may be important, there are other causes.

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COMPLICATIONS OF SMALLPOX VACCINATION IN THE UNITED KINGDOM

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Ever since vaccination was first introduced, many people have been unwilling to accept that it can cause illness and death, and although Jenner (1798)(**9**) stated 'I have never known fatal effects arise from the cow pox', nevertheless the second child selected for vaccination (John Baker, aged 5 years), although free from indisposition on the eighth day, subsequently died of a fever. I am not suggesting that his death was the result of cowpoxing, but Jenner did not even record this death in the classical *Inquiry* of 1798 and it finds only mention in a footnote in *Further Observations on the Variolae Vaccinae* published in the following year(**10**).

There seems no doubt that in the eighteenth and nineteenth centuries many of the deaths associated with vaccination in the United Kingdom were due to infection and were often attributed to negligence on the part of the vaccinees or those looking after them. In the early part of this century the Ministry of Health(**13**) recorded as many as 103 deaths between 1911 and 1922 associated with 4275 109 primary vaccinations, but it was stated that these '*may merely indicate that the child has been vaccinated shortly before death and that death is really attributable to some current illness*'. There was no suggestion at the time that the vaccine might be harmful and it was suggested that if certain simple precautions were taken, the '*procedure* [i.e. vaccination] *can be said to be free of risk*'. Even as late as 1959, one of the most distinguished Medical Officers of Health in the United Kingdom wrote that '*There is virtually no danger from smallpox vaccination if proper precautions are taken*'(**1**), and about the same time an American, in an article on 'Problems associated with routine immunization', wrote that '*smallpox vaccination is an exceedingly safe procedure*; serious complications are rare'(**12**).

COMPLICATIONS

Accurate figures on the number of complications and deaths from vaccination are difficult to assemble, but since 1930 the Annual Reports of the Chief Medical Officer (CMO) have contained data for England and Wales. This was summarized for 1951–60 by Conybeare(**4**). A similar type of analysis was done by Dr John

Barnes for 1961–70 (unpublished) and his data confirm those which I presented to the Committee on Applied Epidemiology in April 1971(7).

The available information on vaccination complications and deaths published in the CMO Reports comes firstly from Reports of Medical Officers of Health about vaccination done in their areas under Local Authority arrangements(3), and secondly from reports of vaccinations performed outside these arrangements, such as in members of the Services and their families, and hospital patients and staff, etc. Since 1964, when the Local Authorities ceased responsibility for notifications of vaccinations in persons of 16 years and over, these reports have included the complications occurring in older persons. It is estimated that excluding 1962, when there was some panic mass vaccination, from 1961 to 1970 about 100000 vaccinations were done each year in that age-group.

DEATHS FROM VACCINATION

Between 1951 and 1970 there were more than 100 deaths from smallpox vaccinations in England and Wales. Seventy-seven of these were reported by local health authorities, 8 by others, and there were 16 deaths from accidental vaccinations. I believe that this total (101) is a considerable underestimate because of under reporting and failure of diagnosis.

In 5·0 million vaccinations, reported in 1951–60 there were only two deaths from accidental vaccination. In 10·6 million vaccinations done in 1961–70 there were 14.

It is not always remembered that in some cases of postvaccinial encephalitis the pathology may be minimal, e.g. perivenous encephalitis does not always occur in children under 2 years of age(5). Spillane & Wells(15) re-emphasized the distinction between postvaccinial encephalomyelitis and postvaccinial encephalopathy. The former does not occur in children under 2 years of age while postvaccinial encephalopathy does occur in infants under 2, as well as in older children. In the latter, the C.S.F. may be normal although under pressure. These cases do not show the characteristic pathology of postvaccinial encephalitis, and although the pathology is not fully known, cerebral oedema and vascular lesions probably play some part. Deaths following convulsions and hyperpyrexia may give very little C.N.S. pathology. I am sure that many of us know of individuals who have had fatal complications which have never been reported.

The age distribution of the 77 deaths reported by Medical Officers of Health under Local Authority arrangements between 1951 and 1970 is shown in Table I.

While it is not always certain whether an adult is undergoing a primary vaccination or a re-vaccination and some of the so-called re-vaccinations in the 15-plus age-group may be primary vaccinations, it may nevertheless be seen from Table I that there is very little difference in the death-rates in the various age-groups except in the under-1-year-old. It has been obvious for some years that in the United Kingdom the death-rates from complications have been highest in small children and in November 1962 it was recommended that elective vaccination should preferably be done during the second year of life. In this respect, it is of interest to compare the death-rates in the under-1-year-olds and 1-year-olds in 1951–60 and in 1961–70 (Table II), which shows a great reduction in the number of deaths in the under-1-year-old children in the last decade compared with 1951–60.

Table I. *Reported numbers of deaths and rates per million vaccinations performed under local authority arrangements in England and Wales from* 1951 *to* 1970

Age-group	Primary vaccinations			Re-vaccinations			All vaccinations	
	No. ('000)	Deaths	Rate	No. ('000)	Deaths	Rate	Deaths	Rate
Under 1	3726	49	13·1	8	0	—	49	13·1
1	2372	7	2·9	13	0	—	7	2·9
2–4	1179	3	2·5	169	0	—	3	2·2
5–14	1465	3	2·0	1224	0	—	3	1·1
15 plus	1912	9	4·7	3597	6	1·7	15	2·7
All	10654	71	6·7	5011	6	1·2	77	4·8

Table II. *Deaths and rates per million for vaccinations in England and Wales in children one year old and under*

Age (years)	1951–60		1961–70	
	Deaths	Rate/ million	Deaths	Rate/ million
Under 1	37	13·9	12	11·3
1–2	2	6·7	5	2·4

Table III. *Causes of death from vaccination in England and Wales,* 1951–70

Complications	1951–60 (no.)	1961–70 (no.)	1951–70 (no. and %)
Post-vaccinial encephalitis	22	18	40
Contact eczema vaccinatum	2	14	16
Vaccinia gangrenosa	7	6	13
Eczema vaccinatum	4	7	11
Benign generalized vaccinia	0	2	2
Others	13	6	19
All	48	53	101

Table IV. *Number of cases of postvaccinial encephalitis between 1951 and 1970 and death-rates per million vaccinations*

Age	No. of vaccinations ('000)	Cases	Rate	Deaths	Rate
Under 1	3734	51	13·7	20	5·4
1–4	3733	32	8·6	8	2·1
5–14	2689*	19	<7·0	2	<0·7
15 plus	5509†	45	<8·2	6	<0·9
All	15665†	147	<9·4	36	<2·3

* From 1965: 5–15 inclusive.
† From 1965: ? +600000.

Of the known deaths associated with vaccination between 1951–70, the commonest causes in each decade are shown in Table III.

POSTVACCINIAL ENCEPHALITIS

This is the most common and most important cause of death. The total number of cases reported between 1951 and 1970 was 151, of whom 40 died, giving a case fatality rate of 26·5%. The age distribution of the cases and deaths reported by local health authorities and rates per million vaccinations in the various age-groups are shown in Table IV.

The numbers vaccinated in the 5–14 age-group are slightly greater than recorded, and the 15-plus age-group should probably be about 6·1 million because, as noted, the number of vaccinations over the age of 15 years was recorded up to 1964 only, and it is estimated that approximately 600000 adult vaccinations were done between 1965 and 1970. These increased denominators would obviously reduce the rates calculated for the older age-groups.

Those who have criticized the abandonment of routine infant vaccination in non-endemic countries have been most concerned about the possible danger of an increased rate of postvaccinial encephalitis if primary vaccinations are done in adults. The numbers and rates of this complication in England and Wales after primary vaccination and after re-vaccination are shown in Table V. These figures indicate that the complication rate after primary vaccination in adults is greater than after re-vaccination, but in interpreting this rate it must be remembered that the numbers are very small and that the denominators for the 1961–70 period are believed to be greater than usually stated; thus following the outbreak in 1962 over 6 million vaccinations were done, but the accuracy of the reporting of primary or re-vaccination is uncertain. Be that as it may, there is no difference in the death-rates from this complication in primary and in re-vaccinated adults.

In a careful study of the neurological illnesses associated with vaccination, Spillane & Wells(15) found 39 cases which had occurred within 3 weeks of vaccination; of these, 24 followed primary vaccinations and 15 followed re-vaccinations: this gives a ratio of

Table V. *Cases of postvaccinial encephalitis in persons of* 15 *years and over from* 1951 *to* 1970 *after primary or re-vaccination in England and Wales*

(Rates per million vaccinations in parentheses.)

	1951–60		1961–70		1951–70	
Vaccination	Cases	Deaths	Cases	Deaths	Cases	Deaths
Primary	6 (14·7)	1 (2·5)	23 (15·3)	1 (0·7)	29 (15·2)	2 (1·0)
Re-vaccination	8 (7·9)	3 (3·0)	8 (3·1)	1 (0·4)	16 (4·4)	4 (1·1)
All	14 (9·9)	4 (2·8)	31 (7·6)	2 (0·5)	45 (8·2)	6 (0·9)

1·6 : 1·0 for neurological complications in primary and in re-vaccinations and is about the same as the ratio of the total cases reported in primary and re-vaccinations in 1951–70 namely 1·8 : 1·0. Of the neurological illness reported by Spillane & Wells(**15**) there were 14 patients with encephalitis or encephalomyelitis; 7 with meningism (an unusually severe, 'but probably common, reaction to the viraemia of vaccinia'), 3 in whom epilepsy was initiated or provoked, 6 with primary focal lesions or with a relapse suggestive of multiple sclerosis, and 9 in whom the peripheral nervous system was involved.

Of the patients with encephalitis or encephalomyelitis studied by Spillane & Wells, 7 were in primary vaccinated and 7 in re-vaccinated individuals; 10 recovered fully, 3 had some residua, and there was 1 death which was in a re-vaccinated individual. It is, as I have said, difficult to get accurate denominators, but with a 40 % acceptance rate of primary infant vaccination, to which is added vaccinations done in the Services or for travel, it might be assumed that nearly half of the population studied by Spillane and Wells had had primary vaccination, which would give the same rate of postvaccinial encephalitis after primary vaccination as after revaccination.

In the British Army over the past ten years there have been no deaths from C.N.S. complications of vaccination, and in 2000000 or more primary vaccinations in the United States Army since the Second World War not a single vaccination death has occurred(**11**).

As has previously been pointed out(**6**), re-vaccination involves two risks – that of the primary vaccination and that of the re-vaccination. It can be seen from Table VI that in adults the sum of these risks is about the same as that of the risk of primary adult vaccination: the death risk is about 5 times as great.

There is thus little to support the argument that now that infant vaccination has been abandoned in the United Kingdom we shall run into large numbers of postvaccinial deaths in adults. The data which I have presented put the risks of complications and of deaths from primary vaccination in adults in their proper perspective, but in any event, the vaccination policy which has been adopted in the UK will require very many fewer vaccinations to be done, which will result in fewer complications and fewer deaths.

Table VI. *Rates per million vaccinations of CNS complications and deaths in primary vaccinations and in re-vaccinations in England and Wales, 1951 to 1970*

Vaccination	Cases		Deaths	
	Rates/ million	No.	Rates/ million	No.
Primary: 0–4 years	11·4	83	3·8	28
Revaccination 15+	4·4	60	1·1	4
PV+RV	15·8	99	4·9	32
Primary: 15+	15·2	29	1·0	2

Table VII. *Estimate of cases and deaths from eczema vaccinatum*

Years ...	1951–60		1961–70		1951–70	
Total vaccinations ...	3820369		(?) 12000000		(?) 16000000	
Age	Cases	Deaths	Cases	Deaths	Cases	Deaths
<1	8	4	6	2	14	6
1–	2	0	14	1	16	1
2–	0	0	9	0	9	0
5–	2	0	5	0	7	0
15+	4	0	23	4	27	4
All ages	16	4	57	7	73	11

GENERALIZED VACCINIA

The Ministry of Health Circular 62/48(3) also requested prompt information about 'generalized vaccinia' and death from 'other conditions' associated with vaccinations. In his analysis of the complications reported between 1951 and 1960 Conybeare (4) divided generalized vaccinia into three conditions – eczema vaccinatum and chronic progressive vaccinia, in which the clinical manifestation of generalized vaccinia was in most instances a serious, often fatal illness, and a relatively mild condition which he called mild generalized vaccinia. It was not, however, until 1964 that eczema vaccinatum appeared as a separate heading in the Annual Reports of the CMO.

ECZEMA VACCINATUM

It would seem that in the past this condition has very often not been notified, e.g. during the last outbreak of smallpox in England and Wales in 1962, 28 cases of eczema vaccinatum were reported through the Medical Officers of Health and 8 from other sources, but an additional 43 cases came to light from a

Table VIII. *Cases and deaths from eczema vaccinatum from contact in England and Wales, 1961 to 1970*

	Age					
	1	1–	2–	5–	15+	All
Cases	13	18	27	22	24	104
Deaths	2	3	6	1	2	14

Table IX. *Cases and deaths from vaccinia gangrenosa (chronic progressive vaccinia) in England and Wales, 1951 to 1970*

	1951–60		1961–70		1951–70	
Age	Cases	Deaths	Cases	Deaths	Cases	Deaths
1	8	7	5	4	13	11
1–	—	—	—	—	—	—
2–	—	—	1	—	1	0
5–	—	—	—	—	—	—
15+	—	—	3	2	3	2
All ages	8	7	9	6	17	13

private study and it is unlikely that they were included among those reported through official channels.

A minimal estimate of the number of cases and deaths of eczema vaccinatum from 1951 to 1970 is given in Table VII. The case fatality rate in 1951–60 was 25%, which is twice that for 1961–70 (12·2%): this is perhaps indicative of even greater under-reporting in 1951–60 than occurs at present.

CONTACT ECZEMA VACCINATUM

In addition to eczema vaccinatum in vaccinated individuals, there have been a relatively large number of cases of this condition in the United Kingdom in contacts of vaccinees, as indicated in Table VIII, for the period 1961–70.

I would hazard a guess that the true number is about twice that reported: prior to 1960, contact eczema vaccinatum was rarely reported. There is of course no obligation whatsoever for practitioners to notify these cases.

VACCINIA GANGRENOSA (OR CHRONIC PROGRESSIVE VACCINIA)

This is a rare condition. The number of cases reported between 1951 and 1970 is shown in Table IX.

Most cases occur in young, immunologically abnormal children or in older people being treated with immuno-suppressive drugs.

REDUCTION OF RISKS

CONTRA-INDICATIONS

The numbers of complications can immediately be reduced by observing the contra-indications to vaccination. In the United Kingdom the following are regarded as specific contra-indications to elective vaccination(**8**):

(*a*) Failure to thrive in infants.

(*b*) Exposure to infectious diseases.

(*c*) A history of or the presence of eczema. Infantile eczema is an absolute contra-indication to primary vaccination except in the face of smallpox. Moreover any infant or person with eczema should be kept away for at least 21 days from any recently vaccinated member of the household. Neglect of this advice may give rise to eczema vaccinatum and the risk of death therefrom.

(*d*) Septic skin conditions.

(*e*) Hypogammaglobinaemia.

(*f*) Leukaemia, lymphoma and other reticulo-endothelial malignancies.

(*g*) Corticosteroid and other immuno-suppressive treatment, including radiation therapy.

(*h*) Pregnancy.

RISKS OF VACCINATION VERSUS RISKS OF SMALLPOX

In the United Kingdom, variola major ceased to be endemic in 1935. During the past 20 years there have been 13 importations giving rise to 103 indigenous cases and 37 deaths, i.e. about 3 deaths per importation. During the same period (1936–70) there were four importations of variola minor with no deaths. Now, during the past 20 years, there have been more than 100 deaths from vaccination, and if things remain the same, we could expect 150 vaccination deaths in the next 30 years. To outweigh the expected 150 deaths from vaccination from now until the end of the century there would have to be about 50 importations of variola major, since each importation has been estimated to cause about three deaths. This is most unlikely on past history, but furthermore, things will not remain the same, for the probability of importing smallpox gets less every year as smallpox becomes eradicated throughout the world.

Dr John Berry (personal communication) has looked at the problem in another way. He has pointed out (Table X) that the median number of importations of smallpox (variola major or minor) was 3 per quinquennium, thus over the next 30 years (i.e. 6 quinquennia) we could expect 18 importations. Now the mean number of indigenous cases per importation has been 12 (with a median of 10), so it could be estimated that the number of cases which would result from importations over the next 30 years would be between 18×12 (i.e. 216) and 18×10 (i.e. 180). From past experience, three-fifths of these would be variola major – say 120 cases which might give rise to 30 deaths; this is to be compared with 150 deaths expected from vaccination in the same period.

While there is always a risk of death from importations, it has been agreed

Table X. *Smallpox in England and Wales, 1936 to 1970*

Quinquennium	All smallpox				Variola major			
	No. of importations originating in the period	No. of imported cases	No. of indigenous cases	No. of indigenous cases per importation	No. of importations originating in the period	No. of imported cases	No. of indigenous cases	No. of indigenous cases per importation
1936–40	9	4 (1)	31 (2)	3·4	7	4 (1)	22 (2)	3·1
1941–45	3	3	24 (3)	8·0	3	3	24 (3)	8·0
1946–50	14	29 (8)	151 (36)	10·8	14	29 (8)	151 (36)	10·8
1951–55	3	1	168 (8)	56·0	2	1	30 (8)	15·0
1956–60	3	3	11 (4)	3·7	3	3	11 (4)	3·7
1961–65	6	8 (2)	62 (25)	10·3	6	8 (2)	62 (25)	10·3
1966–70	3	3	71	23·7	2	3	—	—
Totals	41	51 (11)	518 (78)	12·6	37	51 (11)	300 (78)	8·1

that in the United Kingdom the risk of death from complications is now greater, and as you know, routine immunization in childhood has been abandoned and we can look forward to a reduction in the number of complications and deaths in the next few years.

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Discussion

J. M. LANE (USA) There were at least 19 deaths not caused by postvaccinal encephalitis, eczema vaccinatum or vaccinia gangrenosa. What were most of these deaths?

G. W. A. DICK (UK) These were actually not cases of eczema vaccinatum caused by vaccination but occurring accidentally by contact.

J. M. NEFF (USA) In the study of Spillane and Wells, close to half of the cases of postvaccinial encephalitis occurred in revaccinees. At the time of the study there was massive indiscriminate vaccination of the population over a short period of time. Could these encephalitic deaths have been only temporarily but not aetiologically related to the vaccination? In other words, to what degree did these cases represent the background of neurological illness in that area at that time?

G. W. A. DICK (UK) The difficulty is in knowing exactly how many of the vaccinations were truly primary vaccinations. I think that perhaps people did not always know or say accurately whether or not they had been vaccinated before. My calculations are that Spillane's population was probably 50 % primary and 50 % revaccination.

W. EHRENGUT (West Germany) The incidence of postvaccinal encephalitis in the age-group of 15 years and older in the UK is 14·7 per million primary vaccinations and 7·9 per million revaccinations during the 10-year period 1951–60. This is unusual compared with the figures of West Germany with 1 case in 4·5 million revaccinations. There have been severe cases after revaccination in persons who had a period of at least 40 years between the primary and the revaccination. Now, revaccination at the age of 12 is compulsory in my country. The British death figures for eczema vaccinatum are also extremely high; in West Germany no fatal cases have been observed since 1957, with a vaccination rate of around 800000 primaries a year.

Furthermore, there is no point in looking at the death figures as did Lane. From extensive studies recently published in our book on postvaccinal encephalopathy we concluded that the highest mortality is observed during the first year of life; already in the second year the mortality drops down rapidly. Normally there are no deaths in adults. In contrast, the long-term sequelae are practically zero in babies and highest in adults. So to detect cases of postvaccinal encephalitis in adults one cannot use the death-rate as denominator, but must check the records of neurological and psychiatric departments. Finally, the immunity status of the British population is far better than the 40 %, as stated by Dr Dick. Due to the outbreak of variola in 1962 over 3·2 million persons received a primary vaccination. This is a good support for the national immunity status. Moreover, such a group of recently vaccinated youngsters is very important for the prevention of spread of smallpox.

G. W. A. DICK (UK) The problem is: what is a primary and what is a revaccination? Does an individual vaccinated as a baby react as a primary or as a revaccination when vaccinated for the second time at the age of 40?

I do not think I said that children were responsible for spreading smallpox in the UK. In recent years the important spread has originated around hospitals. The cases of eczema vaccinatum are giving us considerable concern. Careful observation of the contra-indications to vaccination could have prevented these cases.

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COMPLICATIONS FOLLOWING SMALLPOX VACCINATION

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The risks of smallpox vaccination in the United States were poorly documented until Greenberg(2) studied vaccination complications during a mass vaccination campaign in New York City in 1947. Greenberg's work had three defects. First, the tabulation of vaccinations may have been subject to error because of the emergency nature of the vaccination campaign. Secondly, vaccinations were not separated by age of the recipient, nor were primary and revaccinations distinguished. Thirdly, patients with vaccination complications were detected through passive surveillance and search of hospital records. Thus, the 'minor' complications were not well studied.

During the late 1960s the Smallpox Program of the Center for Disease Control conducted studies to estimate the nature and extent of vaccination complications in the United States. This paper will summarize these studies, and emphasize the conclusions reached by American public health authorities regarding vaccination and vaccination policy.

VACCINES AND VACCINATIONS

VACCINES

In the United States all smallpox vaccine is made by seven licensed manufacturers. Essentially all vaccine is either lyophilized or distributed as glycerinated calf lymph. All American manufacturers grow their vaccine on cows, and all use seed strains derived from the New York City Board of Health strain. In our studies of vaccination complications, we did not distinguish between the different vaccine manufacturers, or between glycerinated or lyophilized vaccine preparations.

NUMBER OF VACCINATIONS

The numbers of vaccinations performed annually are not gathered directly. The vaccine manufacturers have voluntarily pooled information on the number of doses distributed in the United States. These figures do not help to determine the numbers of vaccinations by age and previous vaccination status. Starting in 1963, however, a National Immunization Survey has been done annually to estimate the number of immunizations against smallpox and other diseases. This survey is conducted among a sample of over 35 000 households drawn by

Table I. *Vaccinations in the United States, 1968, by age and vaccination status*

Age (years)	No. of vaccinations
Primary vaccinations	
<1	614000
1–4	2733000
5–9	1553000
10–14	295000
15–19	111000
20+	288000
Unknown	
Total	5594000
Re-vaccinations	
<1	0
1–4	478000
5–9	1643000
10–14	1440000
15–19	1217000
20+	3796000
Total	8574000

the Bureau of the Census. The standard error of estimate of the number of vaccinations performed in each age-group is less than 1%. The total number of vaccinations estimated by this method agrees very closely with the total number of doses of vaccine distributed by the various vaccine manufacturers (**5**, **9**).

Table I presents the numbers of smallpox vaccinations by age and vaccination status for the calendar year 1968.

COMPLICATIONS

DETECTION OF VACCINATION COMPLICATIONS

Complications of vaccination are not reportable in the United States, nor is there a regular programme of surveillance for these conditions. Encephalitis is a reportable condition, but is considered to be poorly enumerated. For these reasons, we decided to use more active forms of case detection.

Recipients of Vaccinia Immune Globulin Following considerable publicity about the existence of vaccination complications during the late 1950s, the American Red Cross in conjunction with Professor C. Henry Kempe developed a system to distribute Vaccinia Immune Globulin (VIG) throughout the United States (**3**). In 1964 the recipients of VIG in 1963 were contacted through their physicians, and pertinent data were gathered on each patient (**9**). In 1968 physicians requesting VIG were contacted shortly after their request, regardless of whether or not VIG was released for their patients (**5**).

Case reporting from state and local health departments Cases of postvaccinial encephalitis may be reported to the Center for Disease Control. Laboratory specimens for the diagnosis of vaccinia are often referred to the Laboratory Division of the Center for reference diagnosis. These patients were investigated and included in the 1968 study (**5**).

Death certificates Death certificates with causes of death mentioning vaccinia were used to identify fatal vaccination complications. We have found in the investigation of such deaths that certain patients allegedly dying from smallpox vaccination in fact died from other causes. On the other hand, certain patients dying from vaccination complications did not have vaccinia listed on the certificate (**4**, **5**, **9**).

Vaccine manufacturers and manufacturers of thiosemicarbazone The several manufacturers of smallpox vaccine, plus the sole distributor of thiosemicarbazone, were asked in 1968 to provide information on vaccination complications which came to their attention.

Direct mail surveys of physicians From preliminary analysis of the national surveillance data, and the knowledge of the habits of practising physicians, we were confident that more direct methods would be required to detect clinically mild complications, particularly accidental implantations, generalized vaccinia, erythema multiform, etc. For this reason, we conducted direct mail surveys of physicians in selected states during 1963 and 1968 (**1**, **6**, **7**, **10**, **12**, **13**).

During 1964 and 1965, questionnaires were mailed to 4900 physicians in four states. Replies were received from nearly 91 % of these physicians. The four stated included approximately 4·5 % of the United States population. We found that the clinical and epidemiologic details of many of these 'mild' cases had been lost in the year or two between their occurrence and the receipt of the questionnaire. Therefore, late in 1967, we contacted all physicians in ten states representing nearly 12 % of the United States population, and requested that they record information on vaccination complications. In late 1968 and early 1969, these physicians were surveyed, and responses obtained from 14979 of them for a response rate of 83·9 % (**6**, **9**, **10**).

These direct mailings were designed to detect physicians who had treated patients with suspected vaccination complications. Each physician who reported having seen such a case was contacted again in person, by telephone, or by letter to gather full clinical details about the patient.

PEDIATRIC CLINIC STUDY

Perhaps as high as 1 % of all primary vaccinees suffer a mild erythema multiform, or erythematous urticarial rash, some 5–10 days after vaccination. In order to estimate the actual incidence of these rashes, a prospective follow-up of a cohort of vaccinees at a large pediatric clinic was performed (**8**).

DEFINITIONS OF VACCINATION COMPLICATIONS

The terms and definitions of vaccination complications found in the world's literature are variable. We do not claim that our definitions are necessarily the best. Our definitions were elaborated by Dr John Neff and others in 1963(**9**), and we have used them ever since in order to maintain comparability from one study to another (see Appendix).

RATES OF VACCINATION COMPLICATIONS

Using the numbers of patients with vaccination complications detected through the several sources, and the estimates of the numbers of vaccinations obtained by the United States Immunization Survey, we have calculated the occurrence of various complications by age and vaccination status. Table II represents our best estimate of the rates of complications of vaccination in the United States.

The rates for the rare or uncommon complications, postvaccinial encephalitis and vaccinia necrosum, are taken from the 1963 and 1968 national surveillance data. Because the more common and less severe complications, particularly eczema vaccinatum, generalized vaccinia, and accidental implantations were much higher in the state surveys (from 5–20 times the national surveillance rate), we have used the rates taken from the 1968 state survey data. The rate for the erythematous urticarial reactions is taken from the longitudinal study of pediatric primary vaccinees. Because the policy in this clinic was to vaccinate children during the second year of life, rates cannot be estimated for the age-groups under 1 year of age and over 4.

The death-rates require additional comment. We detected nine deaths attributable to smallpox vaccination in 1968, and seven deaths in 1963. While the age distribution of these deaths pointed strongly to the conclusion that the risk of death was much higher in the first year of life than in subsequent years, we felt obligated to expand this series to larger numbers. Accordingly, we studied deaths occurring in the United States from 1959 to 1968, excepting 1967 where data were not available, and reviewed the clinical and pathological records of these cases(**4**). Thus, the age distribution of some 68 deaths was available to us in estimating the 'true' death-rate.

In the United States, until 1971, routine childhood vaccination was pursued vigorously, so that very few adult primary vaccinations are performed. For this reason, we cannot estimate the death-rate among adult primaries. Only one fatality occurred in an adult during the 9 years of death records which we reviewed. If the National Immunization Survey estimates for the number of vaccinations performed in the over-20 age-group can be generalized throughout this whole period, then approximately 3 million primary vaccinations may have been performed in adults during these 9 years. If the adult primary vaccination death-rate was considerably higher than that for children or infants, we should have detected many more adult deaths.

ACQUISITION OF VACCINIA BY CONTACT

Approximately one-fifth of our major vaccination complications in the United States occurred in contacts rather than in vaccinees(**5**, **9**). Obviously, the major category represented is eczema vaccinatum, where contact acquisition of the virus is quite common. These cases are important, since many of the children with eczema vaccinatum are extremely sick. Indeed *all* of the deaths caused by eczema vaccinatum in the United States during our 9-year survey of death certificates occurred in patients who acquired vaccinia by contact(**4**). The rates calculated for risk of primary and revaccination do not include these patients, because in many instances the vaccination status of the child from whom the vaccinia was acquired was not determined. For this reason, the rates of vaccination complications must be taken as *understating* the real risk to the general population from routine smallpox vaccination.

COMPLICATIONS OF JET INJECTION SMALLPOX VACCINATION

There has never been a direct comparison of vaccination by the traditional method and vaccination by jet injection regarding their rates of complication. We carried out a special programme of surveillance of vaccination reactions during an island-wide programme of jet-injection vaccination in Puerto Rico during 1967 and 1968(**11**). During this programme, potential vaccinees were screened for medical contraindications to vaccination, and no children under the age of 1 year, or adults over the age of 60 years, were vaccinated. The methods of detecting vaccination complications were not comparable either to our national surveillance techniques or to the direct mail surveys in our state-survey studies. Therefore, direct comparison of the rates found in this study with those in our mainland US studies is difficult. The rates for all complications were higher than those found by national surveillance techniques but also smaller than those found in our state surveys. We believe that mass vaccination by jet injector is no more dangerous, and may indeed be less dangerous than standard scratch, multiple pressure, or multiple puncture techniques.

COMPLICATIONS OF AVIANIZED VACCINE DELIVERED BY JET INJECTOR

Vaccine licensed in the United States for jet injection must be free of all bacteria. Since it is easier to grow a bacteria-free vaccine on embryonated chicken eggs than on calves, we have been interested in testing avianized vaccine. During the 1967–8 vaccination campaign in Puerto Rico, a number of vaccinations were given with a lyophilized vaccine of egg origin. This vaccine met all WHO criteria, was bacteriologically sterile, and was indistinguishable in standard laboratory tests from regular lyophilized vaccine prepared from calf lymph. During this campaign there were reports of unusually severe primary

vaccination reactions. Fortuitously, certain schools and factories in adjacent towns had been vaccinated at approximately the same time by the same equipment, using calf lymph vaccine in some areas, and avianized vaccine in others. This enabled a comparison of the vaccination responses, with the type of the vaccine as the only major variable.

Only 28% of school children receiving primary vaccination with egg vaccine had a healed, epithelialized lesion 4 weeks after vaccination. Fifteen per cent of adult primary vaccinees in factories had punched-out ulcerating lesions 28 days after primary vaccination. Whereas all of the calf vaccine children had scars or skin lesions less than 5 mm in diameter at 4 weeks postvaccination, over 90% of the children receiving egg vaccine had lesions larger than 5 mm. Vaccinia virus was isolated from 3 of 24 swabs taken from patients with such lesions 4 weeks after vaccination, whereas no patients vaccinated with calf lymph vaccine shed virus that long.

CONCLUSIONS

The study of the risk of smallpox vaccination complications yields information concerning the contraindications of vaccination, the risks of a policy of routine vaccination, and the pathogenesis of complications themselves.

The risks associated with primary vaccination are greatest during the first year of life. The age-specific differences are particularly pronounced when the life-threatening illnesses are considered. While there is some increased risk with increasing age, the United States data do not show a marked increase of risk in adults over infants.

Revaccination carries extremely low risks. We have not systematically gathered data on the length of time between prior vaccinations and revaccinations which result in complications, but it is our impression that most complications occurring in revaccinees occur only when patients have had 15 or more years between vaccinations.

The efficacy of treatment regimens for vaccination complications is difficult to estimate. We believe that treatment with Vaccinia Immune Globulin and the thiosemicarbazone derivatives benefits patients with eczema vaccinatum and vaccinia necrosum.

From a statistical point of view, eczema vaccinatum is more severe in patients who acquire vaccinia by contact than in those who acquire it by vaccination. In endemic areas where vaccination is necessary, serious consideration should be given to carefully vaccinating children with eczema rather than leaving them unvaccinated. We have no known instance in which vaccination of an eczematous child given VIG simultaneously has resulted in eczema vaccinatum.

The risks of routine vaccination are very small, and may be reduced by prudent observation of known contraindications. The occurrence of complications can be used as a justification for discontinuing routine vaccination *only* if the risk of smallpox importation is low, and a capacity to handle importations is well developed.

Table II. *Complications associated with smallpox vaccination per* 1 000 000 *vaccinations*

Age at vaccination	Complications per million primary vaccinations					Complications per million revaccinations
	<1	1–4	5–19	20+	All ages	All ages
Death (from all complications)*†‡	5·0	0·5	0·5	Unknown	1·0	0·1
Postvaccinial encephalitis†	6·5	2·2	2·6	3·5	2·9	0·0
Vaccinia necrosum†	0·0	0·4	1·0	6·9	0·9	0·7
Eczema vaccinatum§	14	44	35	30	38	3
Generalized vaccinia§	394	233	140	212	242	9
Accidental infection§	507	577	371	606	529	42
Erythematous urticarial reactions‖	Unknown	9600	Unknown	Unknown	Unknown	Unknown

* Neff *et al.*(7).
† Lane *et al.*(4).
‡ Lane & Millar(5).
§ Lane *et al.*(6).
‖ J. M. Neff (unpublished data).

SUMMARY

The United States Public Health Service has conducted a series of studies of the incidence and nature of vaccination complications. These studies were designed to estimate the influence of age and vaccination status on the risk of vaccination. Our best estimate of these risks is given in Table II. There are indications that jet injection of vaccinia virus carries approximately the same risks as does traditional vaccination techniques. Jet injection of avianized vaccine is followed by an unacceptably high rate of ulcerated and slowly healing primary lesions.

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Discussion

G. EDSALL (UK) I would like to clarify one quantitative statement towards the end of your conclusion. You know no instance in which vaccination in an eczematous child given Vaccinia Immune Globulin (VIG) simultaneously has resulted in eczema vaccinatum. Am I correct in interpreting the Table in your abstract that eczema vaccinatum occurs in about 1/125 000 children, and if so about how many children with eczema would one have to vaccinate in order to see eczema vaccinatum?

J. M. LANE (USA) I already mentioned that we do not have a control trial and I am not going to say that we know VIG will prevent eczema vaccinatum. Perhaps Dr Neff wishes to comment on this as he has tried to do a retrospective study.

J. M. NEFF (USA) One question of a study of the incidence of eczema or atopic dermatitis in children under 1 year of age in Maryland was whether they had received vaccination. It turned out that 200 children with atopic dermatitis had been inadvertently vaccinated. None of them had eczema vaccinatum and two or three had an erythema multiform-like illness, which is really about the same incidence as in a normal population. So this question is still unanswered. In no study has death from eczema vaccinatum been reported in an inadvertently vaccinated child; all the deaths occurred in contacts with eczema.

J. M. LANE (USA) That is correct. I might also mention that the same is true in evaluating the CV_1-vaccinia strain. Without a control trial the absence of cases of eczema vaccinatum following CV_1-vaccinia does not mean anything because there is no rate to compare it with.

J. A. ESPMARK (Sweden) As to the incidence of complications in Puerto Rico I want to point out that the populations receiving calf and egg vaccine respectively were not really randomized. The other important thing was the use of the jetgun. The Puerto Rico incidence could be compared with that in Brazil, where several million doses of egg vaccine were administered by multiple pressure. The same holds good for Sweden. In the same area no difference in incidence was observed after vaccination with egg and calf vaccine, administered by the same method, namely multiple pressure or scarification. So maybe the use of the jetgun has some influence.

J. M. LANE (USA) We agree with you about Brazil; we are also unaware of any problems in Brazil, using egg vaccine. Probably the jetgun injection has something to do with it because by this method some vaccinia is deposited subcutaneously. In his work in rabbits comparing different vaccines Dr Barker at the Division of Biological Standards observes larger ulcerations only when the vaccine was given subcutaneously. We have no explanation for this finding; we simply present it.

D. A. HENDERSON (WHO) It should be clarified in respect to Brazil that, in fact, virtually all vaccinations were administered by jet injection and that egg vaccine was the principal vaccine employed in Brazil. Reactions of the type reported in Puerto Rico were not observed. Therefore it would not seem reasonable to attribute the reactions to egg vaccine administered by jet injection. There must be some other explanation.

G. J. GALASSO (USA) In our study we used the CAM vaccine and three other vaccines, both percutaneously at 10^6, 10^7 and 10^8 pfu/ml and subcutaneously at 10^5, 10^4 and 10^3 pfu/ml. No differences were observed either in the method or the vaccine. Perhaps the problems seen in Puerto Rico were due to a combination of various factors. It could be the jetgun method, the egg vaccine, the dosage used and even the condition of the arms.

A. S. BENENSON (USA) Do you or anyone in the audience know of any contact infection of hospital patients under immuno-suppressive therapy?

J. M. LANE (USA) We have observed about 35 large vaccination programmes of hospital staff, and we know of no instance in which vaccinia has spread from the staff to a patient with eczema or immuno-suppression. There were, however, various means employed in these hospitals to keep contact between patients and recently vaccinated staff to a minimum.

A. S. BENENSON (USA) I have cultured the vaccination site and on a primary take on a non-vaccinated person there is free virus on the surface of the arm from about the third till the fourteenth day. After revaccination this period is apparently shorter. We have had no secondary spread from a hospital worker to a patient but it is an element of concern.

APPENDIX

Postvaccinial encephalitis Postvaccinial central nervous system involvement, including separately or in combination the following symptoms: meningeal signs, ataxia, muscular weakness, paralysis, lethargy, coma, or convulsions. Note that this definition does not make a distinction between encephalitis and encephalopathy. Nor have we insisted upon elaborate clinical, laboratory, or pathological criteria for the definition of this disease.

Vaccinia necrosum (progressive vaccinia or vaccinia gangrenosa) Spreading necrosis at the site of vaccination, with or without metastatic necrotic lesions occurring elsewhere on the body. We have found that many clinicians confuse this entity with severe normal primary vaccination reactions. In practice, we have insisted that such individuals have evidence of impairment of their immunological systems before we accept the diagnosis. We believe that true vaccinia necrosum is invariably a fatal disease if the patient does not receive appropriate therapy. Thus patients whose lesions heal spontaneously without therapy have not been accepted as cases of vaccinia necrosum.

Eczema vaccinatum Vaccinial lesions either generalized or as individual lesions elsewhere than the vaccination site, in a person who has eczema or a past history of eczema.

Generalized vaccinia Generalized vaccinial lesions that occur in the absence of eczema or other pre-existing skin lesions. Some writers have used the term 'generalized vaccinia' to include eczema vaccinatum, and there are case reports published in the literature which we would consider vaccinia necrosum which have been labelled generalized vaccinia. We have also found that in the absence of careful laboratory work-up or detailed clinical follow-up, it may be difficult to distinguish cases of generalized vaccinia from cases of erythematous urticarial reaction. In theory, we would expect generalized vaccinia cases to have vaccinia virus growing and multiplying in the peripheral lesions. In fact, we have not been able to isolate virus from peripheral lesions in a high percentage of cases. We believe that most of the cases given this label are probably similar in their pathogenesis to the erythematous urticarial reactions.

Accidental infection or accidental implantation Vaccinial lesions resulting from accidental implantation of vaccinia virus in the eye, mouth, or on other parts of the body in the absence of eczema or other pre-existing skin lesions.

Other Vaccinia lesions complicating skin conditions other than eczema (such as acne, poison ivy, sunburn, etc.) in addition to miscellaneous complications not listed above.

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ENCEPHALITIS AFTER SMALLPOX VACCINATION IN DENMARK, 1956–1970

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Vaccination against smallpox is the only vaccination which is compulsory in Denmark, and no child is allowed to enter school unless a valid vaccination certificate is presented. The statistics show that more than 98% have been vaccinated before school age, the last few unvaccinated mostly representing children with contraindications to smallpox vaccination. Some of these are vaccinated later, and at the age of 14, more than 99% of Danish children have been vaccinated against smallpox.

These vaccinations are performed free of charge by the county medical officers, but about 25% of the parents prefer to pay their private doctors to have their child vaccinated.

A special paragraph in the regulations for Danish physicians requires that all reactions from the CNS in relation to smallpox vaccination must be reported immediately by telephone or cable to our Institute, which manufactures the vaccine used. In addition, such complications are notifiable to the central office of the National Public Health Service. It is therefore felt that our data on CNS reactions after smallpox vaccinations are rather complete.

Based on these notifications, I have scrutinized the hospital records for a 15-year period, 1956–70. In many instances the hospitals have follow-up studies on the patients; in other cases I have contacted the family doctor or the family itself to get supplementary information. Altogether the history of 37 notified cases has been surveyed and it was found that 13 of the patients did not have encephalitis and that their illness had no causal relationship to the smallpox vaccination.

It is generally agreed that the diagnosis of post-vaccinal encephalitis is very difficult and that a strictly clinical diagnosis is impossible(1), and it is hardly ever possible with absolute certainty to decide whether a case of encephalitis is due to the preceding smallpox vaccination or not.

This year we did, however, have one case of encephalitis which could be eliminated and it was an interesting experience. One of the public health officers was notified that a child had become ill with encephalitic symptoms 10 days after smallpox vaccination. It so happened that there was no take after the smallpox vaccination of this child, and happily the vaccine could thus be excluded as the

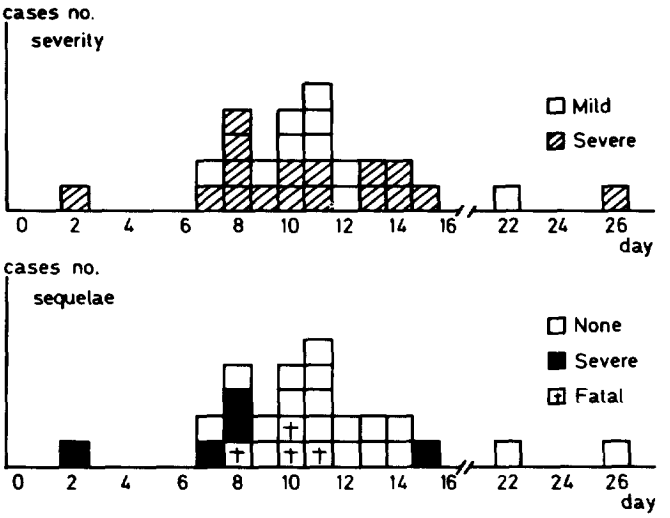

Fig. 1. Interval between smallpox vaccination and encephalitis, Denmark, 1956–70.

cause of her illness. But usually it is a question of more or less probability. In the evaluation it is necessary to include the previous history of the patient, the incubation period, the symptoms and, in case of fatal outcome, the histology.

In 1961 Weber & Lange published an analysis of 265 fatal cases from 1930 to 1960; 136 cases were derived from the literature, while the hospital records for 129 cases were studied by the authors. They found that for children under 24 months of age the shortest incubation period was 4 days, while it was 5 days above the age of 24 months. In this connexion the incubation period is the interval between vaccination and the onset of symptoms from the C.N.S. None of these 265 lethal cases had an incubation period longer than 18 days. Workers of various nationalities almost unanimously agree that the incubation period varies from 4 to 20 days (3, 4).

The interval between vaccination and the onset of symptoms from the C.N.S. for our 27 cases where the symptoms were compatible with a diagnosis of encephalitis related to primary smallpox vaccinations has been recorded in Fig. 1. It will be seen that while the interval for the majority, i.e. 24 patients, was between 7 and 15 days, for 1 patient the interval was 2 days and for 2 patients 22 and 26 days respectively. These 3 patients have been excluded from the study. Two of the 3 cases were severe, but only the case with the 2-day interval between vaccination and illness showed sequelae after the illness.

When we look at the remaining 24 cases, there is a typical distribution of intervals between 7 and 15 days. The severity of illness is shown in the upper part of Fig. 2 and the sequelae in the lower part.

A case of encephalitis has been listed as mild when a patient was ill for only a few days, with fever, convulsions of short duration, some unconsciousness and perhaps temporary slight paralysis and hospitalization for only 1–2 weeks. If a child had more severe reactions, such as prolonged unconsciousness, massive

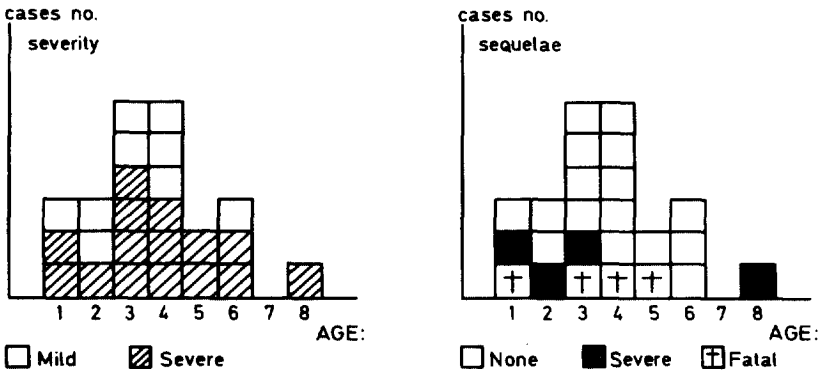

Fig. 2. Age at vaccination and severity of encephalitis, Denmark, 1956–70.

convulsions and pronounced paralysis and prolonged hospitalization, it has been listed as severe.

As regards the sequelae, it was found that the patients in the present study either recovered completely or had severe sequelae (or died).

There is perhaps a correlation between a short interval and the severity of illness, in that there were 6 severe cases out of 12 between day 7 and 10 and only 2 severe cases out of 12 with intervals between 11 and 15 days. However, this difference is statistically not quite significant. The findings have been summarized in Table I.

Sixteen of the patients showed complete recovery. One child had a slight speech disorder afterwards. However, this child had already some difficulties before his illness and had had training with a speech therapist. He has therefore been included in the complete recovery group.

Fig. 2 shows the age of the children with C.N.S. reactions. In Denmark children are hardly ever smallpox-vaccinated until the second year of life, and as they have to be vaccinated before entering school, the age distribution from 1 to 6 years of age could be expected.

As regards the 8-year-old child, there is in the hospital record no explanation for the delayed vaccination of this child, for instance no information about contraindications for vaccination. This particular child is now very severely handicapped, and for the present study it has been decided not to explore the pre-illness history of this child further.

For the period in question, 1956–70, we do not unfortunately have information about the age distribution of the children at the time of vaccination, and our data do not therefore allow any additional information about age disposition for complications after smallpox vaccination of children (**2**).

In the literature there is occasional reference to the tendency of smallpox vaccine reactions to appear in clusters within a relatively short period or within families. We have not observed such a tendency. Fig. 3 shows the occurrence of cases during the 15-year period, 1956–70. Only 1960 and 1970 have been completely free from cases. It might look as if 1968 showed an accumulation of

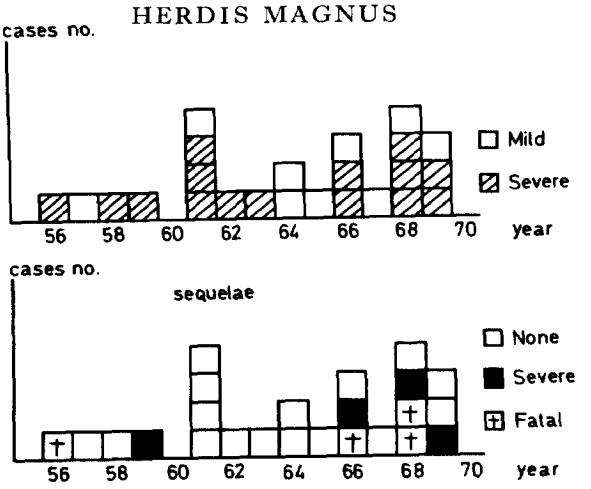

Fig. 3. Distribution of postvaccinal encephalitis, Denmark, 1956–70.

Table I. *Encephalitis after smallpox vaccination, Denmark, 1956–70*

		Sequelae		
Cases	Number	None	Severe	Fatal
Mild	9	9	—	—
Severe	15	7	4	4
Total	24	16	4	4

Table II. *Postvaccinal encephalitis Denmark, 1956–70, four fatal cases*

Case no.	
1/56	Severe case (1956)
1/66	Died during lumbar puncture
3/68	Hydrocephalus prior to vaccination
4/68	Pancreatitis prior to vaccination

Table III. *Smallpox vaccination, Denmark, 1956–70, four patients with severe sequelae*

Patient no.	
1/59	Severe mental deficiency, oligophrenia
5/66	Mental defects, slight paralysis of left side
1/68	Mental defects, paralysis of legs
2/69	Mental retardation, epilepsy

Table IV. *Postvaccinal encephalitis, Denmark, 1956–70, 1·2 million children vaccinated*

	Number	Rate	Number per million
Encephalitis cases	24	1:50000	20
Sequelae and deaths	8	1:150000	7
Deaths	4	1:300000	3:3

particularly severe reactions. However, one of the two fatal cases had hydrocephalus before vaccination, and the other child had not been quite well for a month before vaccination. On autopsy it was found that this child had been suffering from pancreatitis (Table II).

As regards the two other fatal cases, one child died suddenly during spinal

Table V. *Postvaccinal encephalitis*

	England and Wales, 1961–70	Denmark, 1956–70	USA, 1968
Children			
Age in years	< 15	1–7	< 10
Vaccinations	5 329 500	1 200 000	4 900 000
Encephalitis	48 (12)	24 (4)	15 (4)
Encephalitis per million	9 (2·2)	20 (3·3)	3 (0·8)

puncture, and the child who died in 1956 would probably have survived if the therapeutic measures available at a modern hospital today had been applied.

The four children with sequelae are all very severely handicapped (Table III). The defects in case no. 2 may possibly be ascribed to brain damage due to prolonged anoxia in connexion with a pneumothorax, which developed during positive pressure ventilation.

It can be estimated that 1·2 million children were vaccinated during the 15-year period dealt with in the present study. This gives a rate of 1 case of encephalitis per 50 000 vaccinated or 20 cases per million. For sequelae or death the corresponding figures are 1 per 150 000 and 7 per million (Table IV).

How do these figures compare with data reported from other countries?

Labre and co-workers(**5**) from France published in 1964 a comparison of observations made in various countries and the data have been included in a study by McNair Scott(**6**).

The figures for postvaccinal encephalitis were lowest for Britain, 1951–8, with 15 cases per million, and highest for Austria, 1948–53, with 300 per million vaccinated. For Britain the figure was based on $3\frac{1}{2}$ million vaccinated while Austria had 213 000 vaccinations. A later publication from Austria(**1**) gives the information that the number of fatal encephalitis cases (and presumably also the non-fatal encephalitis cases) have been reduced most considerably in Austria during the last decade. Table V shows more recent data from England and Wales and from USA.

For obvious reasons it is difficult to make a direct comparison of this kind of data. For some countries the data represent minimum figures; from others it represents probably the maximal number of complications.

The present study includes four patients with very severe sequelae. It has been the impression by many workers that postvaccinal encephalitis results either in complete recovery or in a fatal outcome. Of course, such cases with severe sequelae have been reported, but I have not been able to find any tabulation on the frequence with which sequelae occur after postvaccinal encephalitis.

Our efforts to keep a close watch on all cases of suspected postvaccinal cases in Denmark will be continued.

SUMMARY

In Denmark all C.N.S. reactions in relation to primary smallpox vaccination are notifiable by telephone or cable to the Statens Seruminstitut. The present study is based on scrutinizing of hospital records as well as subsequent follow-up of the patients in some instances.

The results have been analysed and compared to corresponding data from other countries.

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Discussion

W. EHRENGUT (West Germany) You excluded one case of encephalitis because there was no 'take'. Rehsteiner *et al.* and Fanconi *et al.* demonstrated the presence of antibodies in cases read as unsuccessful. I myself found a serological response in 2 of 100 cases without a local take.

Further, it cannot be excluded that the hydrocephalus you mentioned as a sequela did not exist before vaccination. We demonstrated a statistically significant higher rate of late sequelae after vaccination among children with a pre-existing hydrocephalus or microcephalus or in children who passed through a meningitis. I want to emphasize that these cases can be avoided.

H. VON MAGNUS (Denmark) I will not comment upon the first point. As for your second remark, one has to decide whether to vaccinate or not. We leave this decision up to the parents. If they wanted their child treated like any other child, we think it worth while to take the risk and to vaccinate the child rather than to leave the parents feeling very uncomfortable because their child is so poor that it cannot even be vaccinated against smallpox. Moreover, if you decide not to vaccinate the consequence is a herd of unvaccinated children in a mental institute.

J. M. LANE (USA) I would like to exercise the Chairman's prerogative to mention that to my knowledge I would not vaccinate such a child. There are no data to show that such children have in fact a higher risk and it will be a very hard study to do, but I assume they are.

W. EHRENGUT (West Germany) We can give you these data from our follow-up study. Vaccination of already damaged children results in a statistically higher percentage of damage than in normal children.

G. W. A. DICK (UK) In Denmark the decision is made to continue vaccination of small children. Could we have the intellectual argument as to this decision.

H. VON MAGNUS (Denmark) I do not want to answer this question.

D. A. HENDERSON (WHO) I should like to compliment Dr Von Magnus on her clear and lucid presentations. Next, I have two questions. Your figures for complications are higher than those in the UK and the USA, and perhaps more similar to those in Germany and Austria in the past. I wondered if it has anything to do with the vaccine strain. Have you given any thought to the question of the use of the present vaccine strain?

H. VON MAGNUS (Denmark) I think it might have something to do with the vaccine strain and we are planning to change over to another strain. I also think that these data are not to be compared with other ones. Denmark is a very small country and 75 % of the children are vaccinated by 60 doctors with whom we have a close contact. Moreover, people are very conscious about complications after vaccination and tend to attribute almost anything to the vaccination. So I think our data are nearly complete.

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COMPLICATIONS OF SMALLPOX VACCINATION IN THE NETHERLANDS, 1959–1970

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Since 1959, cases of illness in the postvaccination period, suspected to be complications of smallpox vaccination, are reviewed by a Panel under the auspices of the National Health Council. Formerly, only suspect cases of post-vaccinal encephalitis were reviewed. Neither the classification scheme nor the procedures were changed during the 12-year period. Cases were reported either by the medical attendant or the laboratory, or through the causes of death registration. Such complications have never been notifiable, but the basis of voluntary reports should not be considered as less efficient in this country. Perhaps reporting was somewhat more complete during the later years of the period under review. Completeness is, of course, not claimed.

Most data on infant vaccination will be presented in two 5-year periods, 1959–63 and 1964–8, and a third 2-year period, 1969–70, for which the information is provisional. The tabulated data can be broadly related to equal numbers of annual primary vaccinations, about 210000 for infants and 3000 for adults, including children of 2 years and over.

It should be noted, however, that changes in vaccination policy may have affected the frequency of complications:

(1) After 4 years, from 1 January 1963, the less-pathogenic Elstree vaccinia strain(**2**) replaced the Copenhagen strain in the RIV smallpox vaccine.

(2) It became customary in the course of years around 1962 to administer 2 ml of vaccinia-immunoglobin 16% concurrently with primary vaccination of *adults*. This substance had shown an effect, about 70%, in a trial in recruits, with regard to prevention of encephalitis postvaccinalis(**1**). Histologically, this disease is characterized by perivenous demyelination and microglial proliferation. Postvaccinial cerebral disease in *infants* has a different substrate: brain oedema, ganglion cell degeneration and only minor perivascular infiltration. This encephalopathy is far less specifically a vaccinia complication than perivenous microglial encephalitis(**3**). Vaccinia immunoglobin has not been applied in infant vaccination.

(3) While in the early years of the period infant vaccinations were usually performed in 2-month-old infants, in later years the age of vaccination shifted to the third and fourth quarter. However, this age trend was not universal and cannot be substantiated by figures. Details on vaccination age within the first year of life have been collected only recently.

These changes refer to comparisons between the two 5-year periods. The last 2 years, 1969–70, might be considered as a continuation of the preceding quinquennium.

Table I. *Infant vaccinations – outlines of classification by Review Panel of 522 reports from 2 530 000 vaccinations*

	Reviewed cases	Fatal cases
Vaccinia virus infections		
In vaccinees	43	9
In others	158	3
Encephalopathy	50	27
Other diseases		
Of CNS	20	7
Non-CNS	10	8
Dubious, but vaccination/death relation		
Not to be rejected, onset within 19 days	16	16
To be rejected, late onset	4	4
Other dubious cases (late onset or not fatal)	10	3
Slight untoward reactions to vaccination	104	0
Unrelated to vaccination	107	11
Total	522	88

INFANT VACCINATION

The Panel reviewed 522 cases (Table I) reported as potential consequences of infant vaccination. Twenty per cent (107) were held to be unrelated to vaccination and again 20 % (104) as minor reactions to live virus inoculation. In the remaining 311 cases, the occurrence of a pathological condition in the post-vaccinal period may be anything from a fortuity to a direct causal relationship.

Vaccinia virus infections in vaccinees or their contacts and in others who were directly exposed to the virus (201 cases) are unquestionable complications. However, the clinical diagnosis may be wrong, and in our series the complication which was suspected in three patients was due in fact to herpes simplex virus.

Encephalopathy (50 cases) may occur as well in unvaccinated infants, and this point has even more strength for a variety of other paediatric conditions, whether localized in the CNS (20) or not (10).

In 30 cases the Panel admitted to be unable to make a proper classification. According to the rules, such cases were not rejected but acknowledged as a separate group (16) if the outcome was fatal and the onset of the complicating illness came within 19 days from vaccination. The data were either incomplete or ambiguous.

Complicating *vaccinia infections in vaccinees* were markedly most frequent in the early period (Table II). Benign generalizations (21) were registered only for the Copenhagen strain and though a few vesicles – allocated by the Panel to the

Table II. *Infant vaccinations complications by vaccinia virus (Deaths in parentheses)*

Period	1959–63	1964–8	1969–70
In vaccinees, totals	35	5	3
Normal skin			
Benign generalizations	21	—	—
Immunodeficiency			
In males	5 (4)	—	1 (1)
In females	2	—	1
Vulnerable skin	7 (2)	5 (2)	1
In contacts, totals	97	46	15
Normal skin or mucosa	73 (1)*	21	9
Vulnerable skin or mucosa	24	25 (2)	6

* Foetal death (*Lancet* **i**, 258, 1961).

Table III. *Infant vaccinations – vaccinia virus infections in contacts*, 1959–70

Intra-household infections	
Mother	77
Father	16
Both parents (venereal infection in 3 of 8 couples)	16 (1)
Brother or sister	22
Grandparents	3
Others	1
Extra-household infections	
In medical setting (hospital, office, health clinic)	
Vaccinators and assistants	11
Infants	7 (1)
Nurse in hospital	1
First aid (foreign body in eye) patient	1
Elsewhere	3

group of 'slight untoward reactions' – may emerge if the Elstree strain is used, there is no doubt that in this respect Table II presents an effect of the change in vaccine strain. It is, however, less certain whether the decrease in progressive or generalized vaccinia on the basis of immunodeficiency might be a consequence of this strain change. Here, a shift to higher vaccination ages may have been important, as well as a better awareness of the risks.

The major reduction of *vaccinia in contacts* with normal skin and mucosa, from 73 cases in 5 years to 30 in 7 years, should be attributed, in the first place, to the higher vaccination coverage of the infants' parents when they were still young, but virtual disappearance of benign generalization with the change of virus strain may have contributed. Vaccination rates were very low for young children between 1928 and 1940. As may be noted from Table III, the infant's

Table IV. *Infant vaccinations – occurrence of encephalopathy (Deaths in parentheses)*

	1959–63	1964–8	1970–1
Vaccinations (× 1000)	1033	1071	424
Cases confirmed by autopsy	13 (13)	9 (9)	1 (1)
Clinical diagnosis	21 (4)	5	1
Case fatality by age and sex			
	1–3 months	4–12 months	Total
Male	11/16	7/17	18/33
Female	5/6	4/11	9/17
Both sexes	16/22	11/28	27/50

Table V. *Infant vaccinations – cerebral diseases exclusive of encephalopathy (Deaths in parentheses)*

	1959–63	1964–8	1970–1
Attributed to:			
Bacteria	4 (2)	2 (2)	1
Virus	7	2	1
Toxoplasma	1 (1)	—	—
Other causes	2 (2)	—	—
Totals	14 (5)	4 (2)	2

mother is particularly at risk and the mothers of babies of the later period were undoubtedly better vaccinated than the mothers of the first quinquennium. Vaccinia in contacts was fatal in two cases of eczema vaccinatum – the father of a vaccinated child and an infant who visited a health clinic that served as well as a centre for vaccination. Furthermore, a mother lost the sight of one eye as a consequence of accidental infection.

Encephalopathy (Table IV) was more frequent in the first 5-year period (34) than in the second (14). Assuming equal numbers of vaccinations in male and female infants, encephalopathy occurred significantly (5 % level) more in boys. Case fatality was highest (chi square = 4·3; $P < 0{\cdot}05$) for infants vaccinated below the age of 4 months. We found no evidence of regional morbidity differences within the country, neither did seasonal differences exceed chance variation. Seven of 23 infants survived with cerebral sequelae (spastic paralysis, oligophrenia, epilepsy).

Other diseases of the CNS also were most often registered for the early period (Table V). In 18 of 20 cases a microbial cause was proven or seemed probable. Although all these cases represented known disease entities, the Review Panel

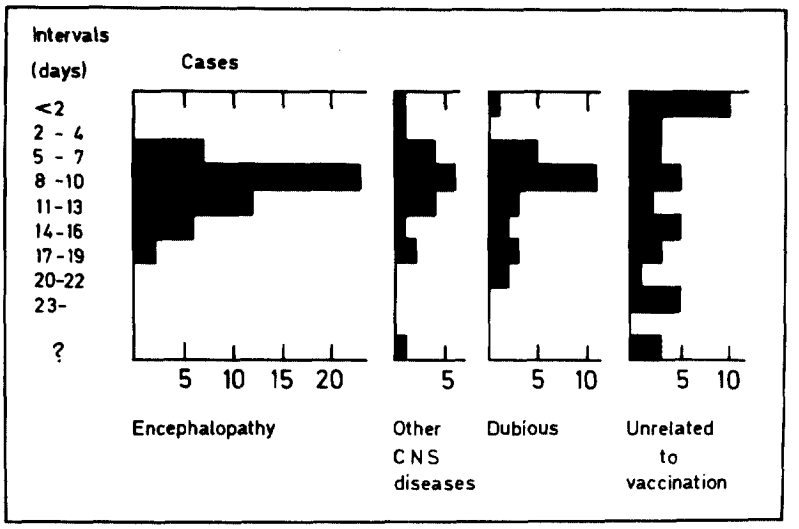

Fig. 1. Interval between vaccination and onset of illness in infants.

considered that the vaccinia infection might have provoked or aggravated the concurrent illness.

Table I shows 10 cases of non-CNS disease; in all 8 fatal cases autopsy reports are available. Three infants suffered from lower respiratory infection, 2 from dyspepsia, 2 from the haemolytic-uraemic syndrome, and 3 from, respectively, myocarditis, staphylococcal septicaemia and hepatitis.

As a true complication may be characterized by a particular distribution of the 'incubation' period, the interval distribution is shown in Fig. 1 for four categories. The 'dubious' and 'unrelated' categories are formed by selected cases in which cerebral involvement was suggestive, but including all fatalities.

For encephalopathy, the distribution is a perfect model indeed for a true complication. For the 'unrelated' group, the opposite holds, as it should. Both other groups have a somewhat suspect mode at the 8–10 days interval and the 'dubious' group in particular may include more than mere coincidences. However, we must realize that for all groups bias may have interfered in reporting as well as in the Panel's allocations, adding undue weight to the second week after vaccination.

Though the official causes of death statistics cannot provide a better estimate of the frequency of fatal complications in infants, they represent an independent source of information. As Table VI shows, the second period figures more favourable than the first: 16 and 40 fatalities, respectively, after correction for administrative errors. The decrease in deaths due to encephalopathy is also considerable. Furthermore, the cases which were allocated by the Panel to the 'dubious' group represented 30% (12 of 40) of all registered deaths in the early period and only 14% (3 of 21, exclusive of administrative errors) in the later 7 years.

Table VI. *Infant vaccinations – fatal complications in causes of death registration (Central Bureau of Statistics) as allocated by the Review Panel, 1959–70*

Classification by Review Panel	Causes of Death Registration			Surplus of Review Panel
	1959–63	1964–8	1969–70	
Vacciniavirus infections	6	3	—	2
Encephalopathy	16	7	1	3
Other CNS diseases	2	3	—	2
Other diseases	2	—	1	5
Dubious, but vaccination/death Relation not to be rejected	9	2	1	4
Relation to be rejected	3	—	—	1
Death unrelated to vaccination	2	1	2	6
Administrative errors	—	3	—	—
Totals	40	19	5	23

Table VII. *Infant vaccinations – complications in three 4-year periods (Deaths in parentheses)*

	1959–62	1963–6	1967–70
Vaccinia strain	Copenhagen	Elstree	Elstree
Vaccinations	821 000	863 000	845 000
Encephalopathy	31* (16*)	16 (9)	3 (2)
Vacciniavirus infections			
In vaccinees	12 (5)	7 (3)	3 (1)
In contacts	88 (1†)	35 (2)	35

* Inclusive of an infant vaccinated with Copenhagen strain on 9 January 1963.
† Foetal death.

Table VII gives a comparison between three 4-year periods for encephalopathy and for vaccinia infections. The first period refers to the use of the Copenhagen strain and includes for that reason one fatal case of encephalopathy with inoculation date in early 1963. These data seem to be more favourable for the Elstree strain. However, the most recent period shows for encephalopathy a decrease from the 1963–6 rate which cannot be attributed to a change in vaccine production. The decrease in encephalopathy seems, in fact, not a sudden but a long-term phenomenon.

VACCINATION OF OLDER PERSONS (>2 YEARS OF AGE)

While the number of vaccinations performed in infants can be estimated with fair accuracy, the numbers of primary vaccinations and revaccinations, required for international travel and for protection against special risks, are not reported. Our figures are only rough estimates.

Table VIII. *Primary vaccinations (exclusive of infants and armed forces)*

	1959–63	1964–70
Estimates of primary vaccinations	15000	21000
Vacciniavirus infections		
Eczema vaccinatum	—	2
Foetal vaccinia	1 (foetal death)	
In contacts	4	5
Encephalitis postvaccinalis		
Confirmed by autopsy	1*	—
Clinical diagnosis (no deaths or sequelae)	3*	1†
Dubious cases (inadequate data)	2 (2 deaths)	—

* Without vaccinia immunoglobulin prophylaxis.
† With vaccinia immunoglobin.

As regards primary vaccination, the difference between encephalitis postvaccinalis rates for the 5-year period 1959–63 (4 per 15000 inoculations) and the 7-year period 1964–70 (1 per 21000 inoculations) might suggest a true reduction of risks (Table VIII). From earlier sources, the risk of postvaccinal encephalitis after primary vaccination in the Netherlands could be estimated as about 1 in 4000. The 1959–63 data are in agreement with that rate. The recent decrease in risks, if true, might be attributed to the use of vaccinia-immunoglobulin, to the change of vaccine strain, or to a third, as yet completely unknown, cause.

Complications of revaccinations were rarely reported. In two cases acquired immunodeficiency as a consequence of lymphoproliferative disease and cytostatic therapy led to a serious but not fatal complication through progression of vaccinia. These vaccinees planned a journey to smallpox-free countries. They are members of a regrettably larger group of persons whose complications could have been avoided if contraindications had been better observed.

SUMMARY

The results of analysis of complications after smallpox vaccination in the Netherlands during a 12-year period are presented.

Infections with vaccinia virus, either in infant vaccinees or in their contacts (particularly the mother), represent the largest group of complications (201; 11 fatalities, 1 foetal death). Reduction of risks in recent years may be attributed to higher vaccination rates in household contacts and, perhaps, to the change of vaccine strain.

Fifty cases in infants were classified as encephalopathy. The death-rate for 1 to 3-month-old infants (73%) was higher than at the age of 4–12 months (39%). The risks of encephalopathy seem to have decreased gradually during the course of 12 years: 25, 16, 6 and 3 cases respectively in four 3-year periods of about 630000 vaccinations each.

Four cases of encephalitis postvaccinalis (1 fatality) without previous immunoglobin administration occurred during 1959–63 per 15000 (estimate) primary vaccinations of adults. During 1964–70 only one case was reported (with immunoglobulin prophylaxis) per 21000 (est.) primary vaccinations.

Illnesses in 46 infants (31 deaths) were not rejected as complications, but their true nature remains in doubt. They represent either well-known disease entities or the data were incomplete or ambiguous.

Dr W. Nanning, Dr A. van Rossum, Prof. Dr A. Verjaal, Prof. Dr E. de Vries and Dr W. van Zeben served as members of the Review Panel.

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Discussion

J. M. LANE (USA) In opening the discussion on Dr Polak's paper I would like to make the observation that the use of classification groups is very good. In our studies we have the definition of fitting within a certain incubation period and ruling out other illnesses. We have not counted such cases but I think it is much more intellectually honest to do what Dr Polak and his group have done.

W. EHRENGUT (West Germany) The curve of the non-vaccine-related fatal cases shows the same distribution as to the onset of illness as I myself published in the *Müncher medizinische Wochenschrift* of 1969, proving these cases are not caused by the vaccination. Secondly, I very much appreciate your careful interpretation of the various factors which could have attributed to the decrease in complication rate. Maybe there are other influences besides the strain as the same slow drop has been observed in all European countries.

M. F. POLAK (The Netherlands) I have no comment to make.

A. C. HEKKER (The Netherlands) In the Netherlands both lyophilized and liquid glycerinated vaccine is used. Do you know with what kind of vaccine these children have been vaccinated?

M. F. POLAK (The Netherlands) We have no information on this point.

J. M. LANE (USA) I would like to ask whether Dr Henderson has an opinion on this. It is interesting that all these Western European nations have seen some decrease in complications, part of these undoubtedly strain-related. Do you think that just lyophilization alone or the purity of current vaccines may be effective?

D. A. HENDERSON (WHO) I find it difficult to imagine that simply lyophilizing the vaccine would make a difference in the frequency of complications. While freeze-dried vaccine is now being used throughout the world in the eradication programme, there are regretfully few reliable data regarding the frequency of complications in the different countries. Certain limited observations have, however, been made which suggest, even when the relative completeness of reporting is taken into account, that complications are somewhat less frequent in tropical than in temperate areas. Again there seems no reasonable explanation of these differences.

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SMALLPOX VACCINATION BEFORE THE AGE OF THREE MONTHS: EVALUATION OF SAFETY

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The most suitable age for primary smallpox vaccination has been the subject of several studies and numerous disputes, especially during the last decade. Traditionally, the serious complications, such as postvaccinal encephalitis, were thought to increase in frequency with age in a continual fashion. This view was modified on an important point by findings reported from Great Britain by Conybeare(**1**) and from the United States by Neff *et al.*(**12**) and by Lane *et al.*(**9**). These reports indicated an excess risk of serious complications after vaccinations performed before 1 year of age as compared to vaccination later in life.

In none of these studies, however, was it possible to determine if the complication rates varied *within* the first year, although from the data of Lane *et al.*(**9**) it appears that the burden falls mainly on the last half of the first year, at least as far as encephalitis is concerned. The failure to distinguish between the various subfractions of the first year is especially unfortunate since no 1-year period in life is more variable than the first one with respect to physiological and immunological state, etc. Thus maternal immunity, if at all transferred, is present during the first 3–5 months of life but is mainly absent during the second half-year.

The present study is an attempt to compare the risk of complications after smallpox vaccination in the first trimester of life with the risks in other age-groups.

TAKE RATE, FEVER AND IMMUNITY FOLLOWING EARLY VACCINATION

One of the first studies on vaccination in early infancy was reported by Wolff(**13**), who vaccinated newborns and observed a course of reaction that was 'almost always completely afebrile'. Similar findings were reported later by Donally & Nicholson(**2**) and by Malmberg(**10**). Another feature of such early vaccination was an apparently higher resistance in the very young infant as reflected by a relatively low take-rate. Studies by Kempe & Benenson(**7**) and by Doorschodt(**3**) indicated that remaining antibodies transferred prenatally from the mother was the probable cause of this attenuation of the vaccination reaction. On the other hand, the results of Doorschodt(**3**) demonstrated that young infants of

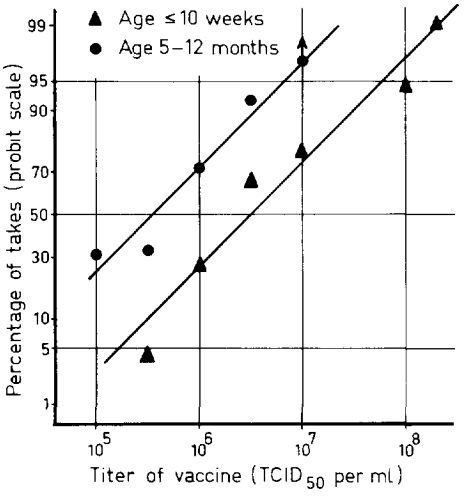

Fig. 1

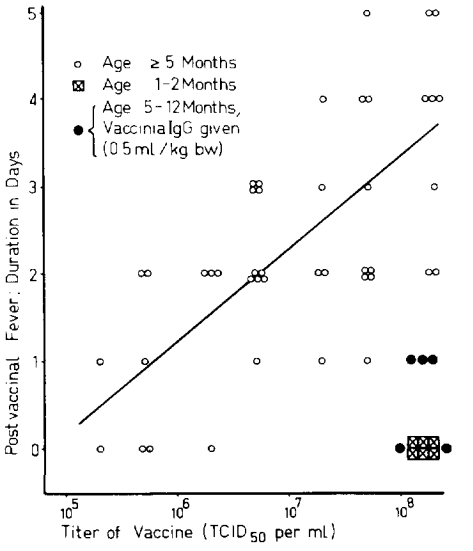

Fig. 2

Fig. 1. Comparison of take-rates in infants aged 10 weeks or less and in children aged 5–12 months after application of a range of dilutions of smallpox vaccine. Success rates, expressed as probits, are plotted against calculated vaccine potencies. The distance between the two parallel curves is almost 1 ^{10}log. (Data from table 2 and figure 1 of Espmark & Rabo, 1965*a*.)

Fig. 2. The dependence of postvaccinal fever reactions upon the vaccine potency and immunity state. Duration of fever in days is plotted against vaccine potency. Most non-immune children exert fever responses which are partially dose-dependent. Note that fever is absent in young infants with maternal immunity and almost absent in older children made passively immune by injection of vaccinia immune globulin. (Data from Espmark, 1965.)

unvaccinated mothers were as sensitive as older children, as indicated by take-rates.

In several studies in Sweden we have tried to find quantitative estimates of some parameters needed for judging the practicability of early vaccination.

Take rates These were compared in infants below 10 weeks of age and in children aged 5–12 months(**5**). As shown in Fig. 1, the pattern of response to the several vaccine doses is similar for both age-groups, but the younger group required approximately 10 times as strong vaccines to yield the same take-rates as obtained in the older group. On the other hand, take-rates in the younger age-group were sufficiently high, i.e. 95 % or more, provided that vaccine titre was adequate (in excess of 10^8 TCID 50 per ml).

Fever reactions Defined as elevation of rectal temperature above 38 °C, fever reactions were almost regularly observed in children aged 5 months or more although a certain dependence upon the dose of vaccine was noted(**4**). This is

shown in Fig. 2. The two small groups of passively immunized infants, i.e. six 1 to 2-month-old babies of vaccinated mothers and five 5 to 12-month-old children given large doses of vaccinia immune globulin, were afebrile or exerted only a 1-day fever response. Also local reactions (pock size) appeared less in the passively immunized groups.

A partial inhibition of the formation of *immunity*, measured as a reduced resistance to revaccination, was noted in infants vaccinated as newborn by Donally & Nicholson(**2**). In Swedish experiments neutralizing antibodies were compared in infants vaccinated at 1 month of age and at 9–12 months respectively(**6**). A possible tendency of lower titres in the younger group could not be shown to be significant.

COMPLICATIONS AFTER EARLY VACCINATION

The findings, in limited trials, of an attenuation of local and fever reactions in infants with maternal immunity does not necessarily mean that the rate of complications is also low in such vaccinees, although this might seem likely. The ultimate proof has to depend on data derived from large samples of vaccinees. However, there is ample evidence that even a moderate passive immunity, produced by injection of vaccinia immune globulin, will significantly reduce the risk of encephalitis after primary vaccination in adults(**11**).

The following data on *vaccination policy* in Sweden are given as a background for the present study. Vaccination was strictly compulsory in Sweden until 1958, when a certain liberalization was introduced. In spite of this only about 90% was covered by childhood vaccination. There was no recommended revaccination in females until 1966, when voluntary revaccination in the 3rd–5th grades of the schools (at the age of 10–12 years) was recommended. In connexion with the 1963 smallpox outbreak in Stockholm about 5% of the population of the country was revaccinated. Other European outbreaks as well as international travel have caused further revaccination. From the data mentioned it may be estimated that 85–90% of the mothers of children covered by this study had a history of primary vaccination and that less than 10% of those had been revaccinated once.

The age of 2 months was officially recommended as the age of choice for routine smallpox vaccination in 1959(**8**); as an alternative the age of 7–9 months was given. These recommendations were further stressed by official circulars and articles in medical journals in 1965.

Earlier this year a *questionnaire* was sent out to vaccine subscribers requesting data on the number of infants vaccinated before 3 months of age, number of takes and number and types of complications. Until now about 50% of the 1100 questionnaires have been completed and returned. Of about 177000 vaccinations reported, about 145000 gave a take. This corresponds to somewhat less than 15% of the total number of children vaccinated. Evidently this figure is an underestimate. The all-over take-rate was 82%. The complications reported on the questionnaires were supplemented by data on officially reportable

Table I. *Reported cases of postvaccinal encephalitis in Sweden, 1961–71: distribution with respect to age*

Age-group	Estimated no. of primary vaccinees (thousands)*	No. of cases of postvaccinal encephalitis
0–3 months	145 +	0
4–12 months	370	5
1–4 years	420	6 (1 death)
5–14 years	215	16 (1 death)
> 14 years	110	38 (1 death)
Totals	1260	65

* The first figure is probably much underestimated; remaining figures, based on data from several sources, are very approximate.

Table II. *Complications following primary smallpox vaccination of 145 000 infants before the age of 3 months*

	Cases
Benign generalized vaccinia	2
Multiple secondary pocks	3
Single secondary pocks	6

complication cases available at the Royal Medical Board. No case of serious complication (postvaccinal encephalitis, vaccinia necrosum or eczema vaccinatum) was reported in the 0–3 months age-group. As seen in Table I several cases of postvaccinal encephalitis were recorded in older age-groups during the time period of this study. There is no significant difference between the 4–12 months group and the 1–4 years group in as far as rate of encephalitis is concerned, i.e. about 1 case per 70000 vaccinees was recorded. With the same rate about two cases would have been expected in the youngest age-group. Clearly, the material is not yet sufficient for any extensive conclusions to be drawn. However, it seems justified to state that the risk of serious complications at the age of 0–3 months does not appear to be greater than at any other age.

All the complications reported in the younger age-group were benign, consisting of secondary spread of single or multiple pocks (Table II).

SUMMARY

Smallpox vaccination in the first trimester of life has been practised on an increasing scale in Sweden for the last 10–15 years. Recently an attempt was made to estimate the rate of complications in this age-group using questionnaires. Among more than 145 000 vaccinees with a take, no case of serious complication was noted. This is less than would be expected if rates were the same as in the latter part of the first year; however, the material has to be further increased before definite conclusions can be drawn.

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Discussion

J. M. LANE (USA) In opening the discussion on Dr Espmark's paper I would simply like to agree with him that in our data in the USA we cannot calculate rates by different months or quarters of the first year of life. We believe that most of the first year of life vaccinations are probably done in the third or fourth quarter. On the other hand, we are dubious that most of our women in the childbearing age have been revaccinated recently enough to have a good titre of maternal antibodies. As there are no actual data on this we are not totally sure that our infants might be protected as they are in Sweden.

J. A. ESPMARK (Sweden) In Sweden revaccination of school children of 12 years of age was introduced in 1966 and none of these women have been involved in this study. Before 1966 there was no revaccination. During the period of study about 5–10 % of the women have been revaccinated, about 90 % had a primary vaccination and ± 10 % had never been vaccinated.

S. S. MARENNIKOVA (USSR) What does it mean, '0–3 months of age': 1, 2 or 3 months?

J. A. ESPMARK (Sweden) Two and 3 months mostly.

H. STICKL (West Germany) The vaccinial immunity in Munich mothers as measured by the haemagglutination-inhibition test is much lower than in Sweden: about 40 % of the mothers of 18–28 years of age and ± 20 % of the 4 week-old-babies had detectable antibody levels.

J. A. ESPMARK (Sweden) We made neutralization tests. If you use the haemagglutination-inhibition test you will miss most of the antibodies. As in a survey 90 % of the mothers were serologically immune and had a scarification, I think our figures are correct.

V. HOCHSTEIN MINTZEL (West Germany) If Dr Espmark's figures concerning the first year of life are added up, the total of five complications is much lower than in any other age-group. This finding is in contrast to all other presentations here.

J. A. ESPMARK (Sweden) My main purpose was to show that vaccination under the shield of maternal immunity would attenuate reactions and perhaps complications too. Although our data are still small they point to the direction that a reduction in complications may be achieved by vaccination at a young age provided that the mother has been vaccinated once in her life.

J. M. NEFF (USA) There are real problems involved in diagnosing a non-fatal case of postvaccinal encephalitis. It is interesting to note that the number of deaths of postvaccinal encephalitis per 100000 primary vaccinations is essentially the same as in the USA whereas the rate of non-fatal cases is far higher than in the USA. This may be due to a lack of accurate diagnostic criteria.

J. A. ESPMARK (Sweden) I do believe that our medical profession is more alert than our honourable American colleagues. In my opinion the difference in non-fatal encephalitis in the US and in Sweden can be attributed to a difference in the efficacy of reporting.

M. F. POLAK (The Netherlands) It may be inferred from observations reported by Swart-van der Hoeven (1964), *Ned. Tijdschr. Geneesk.* **108**, 681, that the maternal vaccination history is not indifferent to the course of vaccinia infection in young infants. Benign generalizations, including also cases with only a few small vesicles which are easily overlooked, occurred more often in infants of unvaccinated mothers (74/128) than in infants of mothers who had been vaccinated in childhood (15/149). The figures refer to infants up to 14 weeks of age, inoculated with the Copenhagen strain.

Session V

GENERAL DISCUSSION

H. VON MAGNUS (Denmark) In response to Dr Henderson's remarks I agree that we should change our vaccine strain. However, the data presented today do not show a significantly higher rate of postvaccinal encephalitis after vaccination with the Copenhagen strain. The figures of death from post-vaccinal encephalitis in the recent years are 3·3 per million vaccinations for Denmark, 2·5 for Sweden and 2·2 for England and Wales, all for comparable age-groups. Moreover, Dr Polak reported a twofold decrease in encephalitis after the change from the Copenhagen to the Elstree strain, whereas a fivefold decrease was observed during the period when the Elstree vaccine was used only.

J. M. NEFF (USA) What are the diagnostic criteria used in defining a case of postvaccinal encephalitis in Sweden.

J. A. ESPMARK (Sweden) I am not a clinician myself but neurological signs of all kinds, connected in time with a smallpox vaccination, are considered as the main symptoms of encephalitis. Disturbances of consciousness is a very important symptom, whereas changes in the spinal fluid are seldom observed. In some countries only death is reported as postvaccinal encephalitis. This is not the case in Sweden; on the contrary, we might have some over-diagnosing.

I cannot resist the temptation to make a comment on the Smallpox Eradication Group. To my knowledge there is not a completely unvaccinated population in which the effect of smallpox importation can be studied. Maybe England will soon be an adequate area for such a study. The situation reminds me of a story. Ten brave but not too intelligent men went out for a long walk in the rain. Of course, they started out with umbrellas. Soon one of them said: 'Now look, we are not getting wet, so let us throw away the umbrellas.' And so they did. It was still raining, so you may imagine the result. This story is in complete analogy with some of the discussions about vaccination policy.

J. M. LANE (USA) It was never said that smallpox vaccination prevents smallpox importation.

G. W. A. DICK (UK) When does Dr Espmark think that Sweden will stop vaccination of infants? Now, in 10 years, or never?

J. A. ESPMARK (Sweden) We will stop vaccinations of infants when the smallpox eradication campaign has advanced so far that there is no fear anymore of importations. During the smallpox outbreak in Stockholm we had 28 cases of postvaccinal encephalitis after primary vaccination of adults. This might not have occurred if they had been vaccinated in childhood.

A. S. BENENSON (USA) I hope that Dr Espmark will succeed in what he is working on, which is vaccination without any adverse effect in the vaccinee. Further, I think vaccination has to be continued until every deep-freezer is emptied of pox virus and until we know that the chimpanzee and cynomolgus monkey pox were purely accidents.

D. A. HENDERSON (WHO) Infant vaccination is now widely practised throughout the endemic areas and the observation is that this seems to be as safe or somewhat safer than vaccination at a later age.

The object of the eradication campaign is hopefully to make vaccination redundant. This campaign protects both the countries with the disease and various countries in Europe and North America against importations. The two European countries that have received most importations during the past ten years did not contribute to the special account for smallpox eradication.

J. M. LANE (USA) In the USA more money is spent on vaccination complications and their sequelae than has been given to the WHO for eradication.

F. DEKKING (The Netherlands) Everybody knows that infant vaccination does not protect non-endemic countries against the spread of variola after introduction. As to the demand for an unvaccinated country, I think Dr Foege has such a country. He did not start a mass vaccination, he started surveillance. That is what had to be done in a non-endemic Western European country.

W. H. FOEGE (USA) Vaccination is not the only protection against smallpox; in some circumstances surveillance and epidemiological control are more important.

SESSION VI

VACCINE DEVELOPMENT ESPECIALLY PERTAINING TO PREVENTION OF COMPLICATIONS

Chairman: Professor C. KAPLAN (UK)
Secretary: Dr G. VAN STEENIS (The Netherlands)

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EVALUATION OF VACCINE STRAINS BY THEIR BEHAVIOUR IN VACCINATED ANIMALS AND POSSIBLE IMPLICATIONS OF THE REVEALED FEATURES FOR SMALLPOX VACCINATION PRACTICE

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One of the factors determining the effectiveness of the smallpox eradication campaign in the world consisted in considerable improvement in the quality of the smallpox vaccine in use, primarily the increase of its potency and stability. Undoubtedly, in the areas where smallpox was or still remains endemic it is these properties that are of foremost importance. At the same time, in the countries free from smallpox for a long time in which vaccination of the population is carried out only to prevent spread of the infection in case of possible importation from outside, other properties of the vaccine acquire substantial importance along with potency and stability. The question is of untoward vaccination-associated consequences in the form of high reactogenicity and possible different, sometimes fatal postvaccination complications. The latter, in particular, were one of the incentives for the recent revocation of routine vaccination against smallpox in England and the USA.

It should be stated, however, that further spread of the tendency to abolish vaccination at this stage, as attested to by the recent experience in Yugoslavia, will hardly influence positively the course and prospects of the global campaign of smallpox eradication. There are reasons to believe that the possibilities of other approaches to overcoming negative aspects of vaccination prophylaxis of smallpox are far from exhausted.

The investigations carried out by us and other authors(**1**, **4**–**6**) have demonstrated that other conditions being equal, the intensity of vaccination reaction is determined by the strain from which a given vaccine has been prepared. With due consideration of this, in the USSR highly reactogenic strains were rejected from production, which resulted on the whole in a considerable reduction of the severity of vaccination reaction in vaccinees.

As for postvaccination complication and particularly postvaccination encephalitis, until recently it was generally recognized that their rate was not associated with the quality of the vaccine strain. Analysis of the relevant data after a less reactogenic strain was used in production in the USSR led us to review this concept(**2**).

Subsequent observations, whose results are presented in Table I, confirm the conclusion that the rate of complications is associated with the quality of the strain used.

Table I. *Rate of postvaccinial complications and severe general reactions after application of smallpox vaccines from different strains*

Strain	Pathogenicity for animals	Reactogenicity	Number of cases per 10 million doses of the vaccine released: Postvaccinial complications, Total	Postvaccinial complications, Encephalitis	Severe general reactions (convulsions)
'Tashkent'	High	High	4·6	1·8	7·0
B-51	Moderate	Moderate	1·7	1·0	0·9
EM-63	Low	Moderate		0·7	

The discovered dependence of reactogenicity of the vaccine and the rate of complications induced by it upon the strain used prompted some experimental studies presented below aimed at the elucidation of the possible mechanisms of these differences in the properties of vaccine strains.

In these experiments, carried out at our laboratory by L. Shenkman, rabbits and monkeys were used as models. They were vaccinated with different strains in an equal dose of 10^6 pfu/0·1 ml usually used for human vaccinations. In these animals the extent of virus multiplication in different organs and tissues during the postvaccination period was determined, clinical manifestations of the process were evaluated and the capacity of the strains to induce interferon at the site of virus application was studied.

The results of the study revealed significant differences between strains in the intensity of virus multiplication in the skin of rabbits after application on the scarified skin and intradermal inoculation and in their regional lymph nodes (Figs. 1, 2). Differences were also found in determinations of the presence of virus in the blood and viscera during the postvaccination period. Thus in rabbits vaccinated on the scarified skin with the EM-63 strain, beside the site of inoculation and regional lymph nodes, the virus was found only in remote (popliteal) lymph nodes in one out of 28 animals 4 days after vaccination. No virus could be recovered either from the blood or from viscera of rabbits. At the same time virus was isolated occasionally from the blood, spleen, liver and lungs at 2–5 days, the brain at 5–6 days and from remote lymph nodes at 7 days in rabbits vaccinated with the Tashkent, Wyeth and L-IVP strains (Table II).

Virus was isolated most frequently from the rabbits vaccinated with the CV-1 strain (blood, remote lymph nodes, spleen, lungs, liver). Distribution of the CV-2 virus in the organs differed little from that of the Wyeth strain except that, in contrast to the latter, the virus was never found in the liver and the brain of rabbits vaccinated with CV-2 but was isolated from the lungs of one out of 16 animals.

In experiments in monkeys vaccinated on the scarified skin, as in rabbit experiments, considerable accumulation of virus was found at the site of inocu-

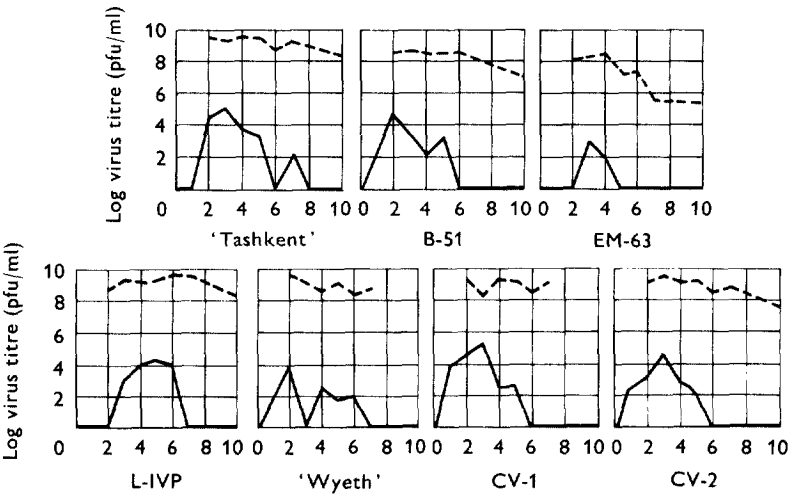

Fig. 1. Dynamics of virus multiplication on rabbit scarified skin (– –) and in regional lymph nodes (——).

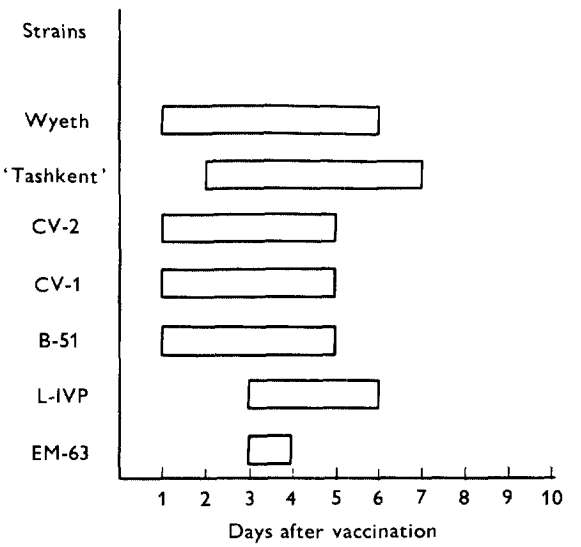

Fig. 2. Duration of virus isolation from regional lymph nodes.

lation and in regional lymph nodes. In contrast to rabbits, monkeys vaccinated with the EM-63 and L-IVP strains showed viraemia at 4–8 days. However more limited circulation of the virus and its distribution in the animals vaccinated with the EM-63 strain were confirmed once again (Table III). In monkeys vaccinated with the EM-63 strain no virus was isolated from the viscera or from the nasopharynx. At the same time, in some monkeys vaccinated with the

Table II. *Results of virus isolation from blood and viscera of rabbits vaccinated with different strains (rate of virus isolation for 3–6 days)*

Material tested	Strains						
	EM-63	B-51	'Tash-kent'	'Wyeth'	CV-1	CV-2	L-IVP
Blood	0/28	0/18	1/18*	1/16	1/16	2/16	0/18
Lymph nodes	1/28	0/18	0/18	1/16†	4/16*	3/16	0/18
Spleen	0/28	0/18	1/18*	1/16	4/16	1/16*	2/18
Liver	0/28	0/18	0/18	1/16	1/16	0/16	0/18
Brain	0/28	0/18	1/18	2/16	0/16	0/16	1/18
Lungs	0/28	1/18	1/18*	0/16	3/16	1/16	1/18
Kidneys	0/28	0/18	0/18	0/16	0/16	0/16	0/18

Numerator: number of rabbits in which virus was detected.
Denominator: total number of animals tested.
* Including isolation on the second day.
† Isolation on the seventh day.

Table III. *Results of virus isolation from monkeys vaccinated with different strains*

Material tested	Strains			
	EM-63	B-51	L-IVP	'Tashkent'
	Rate of virus isolation (3–9 days)			
Nasopharynx	0/8	0/2	3/8	1/2
Blood	2/8	0/2	4/8	0/2
Lymph nodes	0/5	n.d.	0/5	n.d.
Spleen	0/5	n.d.	3/5	n.d.
Liver	0/5	n.d.	1/5	n.d.
Brain	0/5	n.d.	0/5	n.d.
Lungs	0/5	n.d.	0/5	n.d.
Kidneys	0/5	n.d.	0/5	n.d.
	Virus titre on the 6th day (pfu/ml)			
Skin (on vaccination site)	$2{\cdot}3 \times 10^8$	$2{\cdot}1 \times 10^8$	$4{\cdot}4 \times 10^9$	$4{\cdot}6 \times 10^9$
Regional lymph nodes	$1{\cdot}0 \times 10^3$	$3{\cdot}0 \times 10^2$	$1{\cdot}1 \times 10^3$	$2{\cdot}1 \times 10^4$

n.d.: not done.
Numerator: number of monkeys from which virus was isolated.
Denominator: Total number of monkeys examined.

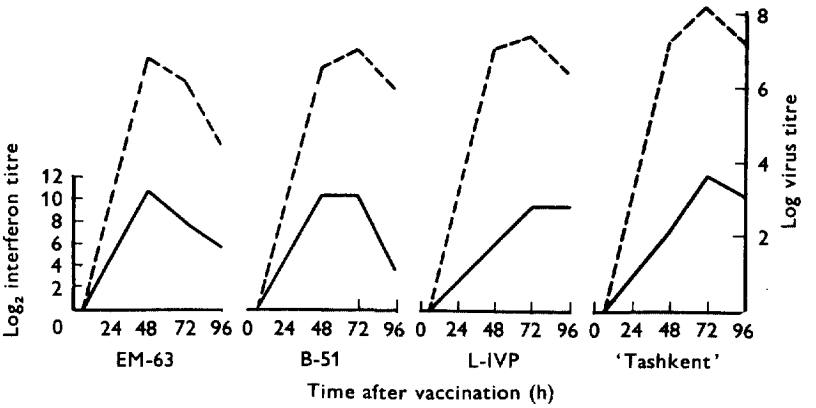

Fig. 3. Dynamics of virus multiplication and interferon production on rabbit skin. – –, Virus; ——, interferon.

L-IVP strain the virus could be isolated from the throat at 3–9 days, from the spleen at 3–5 days and the liver at 4 days.

Interestingly, the clinical manifestations of the reaction in animals vaccinated by scarification with different strains in the above-mentioned dose differed but little. At the same time, it is noteworthy that in vaccinations with the Tashkent, B-51, Wyeth, CV-1 and CV-2 strains maturation of the vaccinal elements in rabbits was more rapid and preceded almost by 24 h that in the animals vaccinated with the EM-63 and L-IVP strains. In rabbits vaccinated with the Wyeth, CV-1 and CV-2 strains a more rapid falling off of the crusts at the site of inoculation was also observed. In monkeys, only slightly more marked intensity of the local process (infiltration, lymphadenites) was observed after vaccination with the Tashkent and L-IVP strains than after B-51 and EM-63 strains.

As has been demonstrated by special studies, differences in the intensity of replication of virus in vaccinated animals given qualitatively different strains correlated with the antigenic and immunogenic activity of the strains(3).

The capacity to produce interferon was determined after intradermal inoculation of virus in the same dose for four of the strains under study (Tashkent, L-IVP, EM-63 and B-51). Simultaneous evaluation of the dermal reaction revealed more clear-cut differences in the character and intensity of the reaction (necroses after inoculation of the Tashkent strain, poor development of infiltration after inoculation with the EM-63 strain) than after vaccination by scarification.

The results of interferon determinations carried out in collaboration with T. A. Bektemirov are presented in Fig. 3 and Table IV. It will be seen in Fig. 3 that the maximum content of interferon in the skin of rabbits coincides with the maximum concentration of virus which begins to decline from the moment that the content of interferon for a given strain is maximum.

In addition to this general regularity, however, certain features of interferon production associated with the interferon-inducing strain were revealed. Thus in rabbits vaccinated with the EM-63 and B-51 strains interferon titres at early intervals (48 h) greatly exceed those in animals vaccinated with the other two

Table IV. *Virus and interferon content in rabbit skin (48 hours after inoculation)*

Strains used for inoculation	Virus titre in skin (pfu/ml)	Titre of interferon (max. and min.)	Geometrical mean
EM-63	$4{\cdot}6 \times 10^6$	640–5120	$1809 \pm 1{\cdot}57$
B-51	$3{\cdot}4 \times 10^6$	640–2560	$1279 \pm 1{\cdot}0$
L-IVP	$1{\cdot}4 \times 10^7$	40–160	$56{\cdot}25 \pm 1{\cdot}0$
'Tashkent'	$1{\cdot}7 \times 10^7$	160	$160{\cdot}6 \pm 1{\cdot}0$

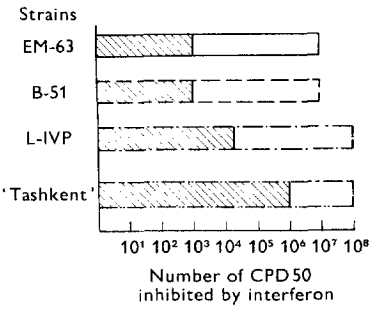

Fig. 4. Interferon sensitivity of different vaccinia virus strains in cell culture tests. Virus titre: □, before treatment by interferon. ▨, after treatment by interferon.

strains. In the latter, despite higher concentration of virus in the skin, interferon is produced less intensively in this period.

Comparison of the results obtained with the pattern of skin reaction in rabbits suggests that it is the early production of large amounts of interferon that may be one of the reasons of the milder local reaction observed after vaccination of animals with the EM-63 strain and to a slightly lower extent with the B-51 strain. On the other hand, delayed accumulation of interferon after vaccination with the Tashkent strain appears to be conducive to more vigorous accumulation and long persistence of virus in the skin in high concentration which, in its turn, manifests in the rate of development of skin reaction and its severity (presence of necroses).

Trying to explain the reason for the peculiar behaviour of the L-IVP strain, which by the pattern of interferon induction was close to the Tashkent strain but produced the skin reaction of lower intensity, we tested the sensitivity of the strains to interferon (Fig. 4).

The L-IVP strain was found to differ from the Tashkent strain by greater sensitivity to interferon. In this respect it was similar to the EM-63 and B-51 strains. It may be assumed that it is this property of the L-IVP strain (that determines lower severity of the local skin process caused by it as compared with the Tashkent strain.

Similar regularities of the interferon-inducing activity of the strains and their pathogenicity were established in tests in another model – white mice inoculated intracerebrally.

Summing up, it may be stated that the investigations performed revealed definite differences in accumulation and distribution of virus in the organism in the postvaccination period with the strains tested. Though the mechanism responsible for these differences is still not quite clear, our data suggest that they may be determined, at least partially, by the capacity of the strains to induce interferon and by their sensitivity to the effect of the latter. For a number of strains (with the exception of the still insufficiently studied CV-1 and CV-2) the established features, in our opinion, may explain the differences in reactogenicity of the strains and partially in the rate of postvaccination complications caused by them.

SUMMARY

The data presented in this report characterize previously poorly investigated properties of vaccine strains, particularly their capacity to multiply in vaccinees, their sensitivity to interferon and interferon inducing capacity.

The strains differed by the duration of induced vireamia and by the spectrum of dissemination in internal organs of rabbits and monkeys.

Considerable differences between strains were revealed while determining their interferon-inducing activity in vaccinated animals as well as the sensitivity of strains to interferon. The strain with reduced capacity to multiply induced interferon production in greater quantity in earlier periods as compared with the strains characterized by high multiplication capacity.

The first were also more sensitive to the inhibiting effect of interferon. Among the strains with high replicative capacity were some sensitive (Lister Institute strain) and some insensivitive to interferon. The most pathogenic and reactogenic strain, 'Tashkent', belonged to the last group.

The data obtained and the results of strain studies carried out earlier will be discussed here from the point of view of smallpox vaccine production as well as smallpox vaccination practice, keeping in mind particularly the problem of postvaccination complication prevention.

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Discussion

HOCHSTEIN-MINTZEL (BRD) (West Germany) Which animal tests would you suggest from your experience should be made to evaluate the pathogenicity of a given smallpox vaccine strain?

MARENNIKOVA (USSR) The tests most commonly used by us are intradermal and intracerebral inoculation of rabbits, intranasal inoculation of 1- to 2-day-old mice and intracerebral inoculation of irradiated white mice.

HENDERSON (WHO) The EM-63 strain appears, from your studies, very satisfactory – perhaps more satisfactory than the others. Could you tell us about the source and history of this strain?

MARENNIKOVA (USSR) We received the strain from Dr Krag-Andersen, Denmark. The strain originally came from Ecuador and is probably similar to the Massachusetts strain, which in 1940 was sent to Ecuador. It was studied and cloned in our laboratory in Moscow in 1963 and was given the name EM-63.

EDSALL (UK) Perhaps I should add a comment or two to the remarks made by Dr Marennikova about strain EM-63. The history of the EM-63 strain very probably *does* go back to Massachusetts. Since the Massachusetts strain has a long history of innocuity it might be worth while to attempt to define the history of strain EM-63 more precisely.

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L-ASPARAGINASE-INDUCED GENERALIZED VACCINIA IN THE RABBIT

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Children vaccinated unwillingly during measles incubation against smallpox sometimes exhibit generalized vaccinia. Since it is known that measles suppress the allergy of the delayed type, we studied the vaccinial allergy in children with generalized vaccinia which was inhibited as demonstrated by intracutaneous tests with non-infectious smallpox vaccine(**2**). The enzyme L-asparaginase shows immunosuppressive activities. Of special interest in this connexion is its inhibitory effect on the blastic transformation of lymphocytes(**3**) by its glutaminase activity(**5**). As a working hypothesis we assumed that due to its attack on the metabolism of the lymphocytes, it might be possible to induce generalized vaccinia.

This report deals with the effect of simultaneous vaccination of rabbits with different vaccinia strains and L-asparaginase treatment.

MATERIAL AND METHODS

Animals One hundred and thirty-nine albino rabbits 'deutscher Riese', an inbred strain of the Vaccination Institute, and 27 albino rabbits 'weisse Neuseeländer' with a weight between 1·75 and 3·95 kg were kept in isolation cages. The animals were fed with approximately 150 g Altromin® daily and water *ad libitum*. The temperature of each animal was recorded twice daily, and the weight was checked on the 1st, 5th, 12th and 19th day of the experiments.

Vaccinia virus The following strains were used: strain 'Elstree' and 'Hamburg', both titre 10^8 pfu/ml (both grown on embryonic calf-muscle cell-cultures); strain 'EM 63' (kindly furnished by Prof. Marennikova/Moscow) in freeze-dried form, titre 10^7 pfu/ml, and in liquid form, titre $10^{7\cdot5}$ pfu/ml; strain 'Bern', titre $10^{6\cdot8}$ pfu/ml (kept for 6 years at −20 °C).

Enzyme L-Asparaginase ('Crasnitin', registered trade mark) donated by Bayer, Leverkusen, Germany. In the course of the experiments different batches were used: (FB b 6366, C 1783: 2 F, G 6, 18 E, 21 E). At the beginning of the trial 2000–5500 u. of Crasnitin were administered daily to each animal. Since no difference in the reactions between the higher and the lower doses could be found, only 2000 u. L-asparaginase (= A-nase) were administered in the subsequent experiments.

To study the *effects of A-nase* in the rabbit 23 animals were treated on 3 subsequent days with intraperitoneal injections. The animals were bled at the onset of the trial, on the 2nd, 5th, 12th and 19th day of experiment, and the serum calcium, inorganic

Table I. *Various effects of i.p.* L-*asparaginase treatment in* 23 *rabbits*

Fever	Hyperpnoea	Conjunctivitis	Loss of weight	Seizures	Chvostek phenomenon
20	6	3	23	8	9

Table II. *Loss of weight (mean weight) in* 23 *i.p.* L-*asparaginase-treated rabbits*

	1st day*	5th day	12th day	19th day
Mean weight (g)	3900	3628	3586	3746
Mean loss of weight	—	−272	−314	−154

* Seven animals died on the 2nd and 3rd day.

Table III. *Mean values of serum calcium, phosphate, alkaline phosphatase, serum protein in* 23 *i.p.* L-*asparaginase-treated rabbits*

	1st day	2nd day	5th day	12th day	19th day
Serum calcium (mg %)	13·7	9·2	11·1	14·2	15·0
Phosphate (mg %)	5·2	8·1	6·6	7·9	7·5
Alkaline phosphatase (mu/ml)	24	34	19	14	24
Serum protein (g %)	6·1	6·51	6·0	5·65	6·07

phosphate, alkaline phosphatase, serum protein, and in a few samples also the kalium, magnesium and lipid content as well as cholesterin, were determined.

In the *second part of the experiment* the vaccination of the animal with the Herzberg method with different strains of vaccinia virus (see above) was performed and immediately afterwards the i.p. A-nase injection followed.

RESULTS

EFFECTS OF I.P. L-ASPARAGINASE TREATMENT OF THE RABBIT

After the first injection of 2000 u. A-nase fever and hyperpnoea occurred in some of the animals (Table I). The second injection of the enzyme was followed 9 times by Chvostek phenomenon on one or both sides of the mouth; sometimes a conjunctivitis, combined with iritis, could be seen. Of 23 animals, 8 developed clonic seizures. The animals were then lying in a lateral position. Due to general lassitude and weakness severe anorexia with loss of weight occurred (Table II) and seven animals died between the 2nd and 3rd day of the experiment. As a cause of death hypoparathyroidism has to be assumed (Table III).

Table III shows a marked decrease in serum calcium on the 2nd day of the

Table IV. *Serum calcium (mean value) in* L-*asparaginase-treated and additional vaccinated rabbits*

	1st day	2nd day	5th day	12th day	19th day
Asparaginase only ($n = 23$)	13·7	9·2	11·1	14·2	15·0
A-nase + 'Hamburg' ($n = 6$)	14·3	7·2	8·7	12·9	14·6
A-nase + 'Elstree' ($n = 27$)	13·9	8·4	9·4	9·6	13·4
Controls ($n = 5$)	14·5	13·0	13·8	12·2	14·9

Table V. *Onset of generalized vaccinia (strain Elstree) in* 18 L-*asparaginase-treated rabbits*

Day after vaccination	3	4	5	6	7	8	9	10	11	12	13
No. of animals with gen. vaccinia	2	1	2	1	1	1	1	2	1	3	3

trial, an increase in phosphate and alkaline phosphatase. The serum protein content remained normal, demonstrating that the decrease in calcium is not due to hypoproteinaemia. Since also the magnesium content of the serum decreased from 2·55 mg % (mean value 1st day) to 1·5 mg % on the 2nd day, the diagnosis of hypoparathyroidism could be established due to the low serum calcium and magnesium and high serum phosphorus level.

EFFECTS OF SMALLPOX VACCINATION AND ADDITIONAL L-ASPARAGINASE TREATMENT OF RABBITS

(*a*) *Strain Elstree* In a first assay, 27 rabbits were vaccinated cutaneously with the Herzberg method (strain Elstree) and treated with A-nase (as described above, batch no. FB b 6366). As in the first trial with A-nase, only the symptoms of the A-nase treatment (Table I) appeared in the beginning, but of the vaccinated animals 23 out of 27 showed hyperpnoea, 13 seizures, 18 Chvostek phenomena. Each rabbit demonstrated a loss of weight and anorexia. Sixteen rabbits out of 27 died between the 2nd and 16th day. Hypocalcaemia was very evident between the 2nd and 12th day of the experiment and the calcium level normalized on the 19th day (Table IV).

It has to be stressed that in the animals vaccinated with the Elstree strain hypocalcaemia persisted up to the 12th day of the experiment. As soon as clinical signs of hypocalcaemia appeared, the animals were treated with i.v. injections of a 20% solution of calcium gluconicum ('Woelm'). To overcome the tetanic condition, 2–34 ml of calcium had to be injected per animal between the 2nd and 5th day of the trial. After this critical phase the immunosuppressive action of A-nase was evident (Table V).

Table VI. *Localization of generalized vaccinia lesions (strain Elstree) in* L-*asparaginase-treated rabbits*

Rabbit no.	Lips	Gingiva	Tongue	Throat	Eye	Stomach	Intestine	Lungs	Orchitis	Anus, penis
20	×	×	×	.	.	.	.	.	×	.
23	.	×	.	×	.	.	.	.	.	×
24	.	.	×	.	.	.	.	.	×	.
26	×	×	×	.	.	×	.	.	.	.
27	×	.	×	×	.	×	×	.	.	.
29	×	×	×	×	×	×	.	×	.	.
30	.	×	×	.	.	.	.	.	.	.
31	×	×	×	×	.	.	.	.	.	×
32	.	.	×	×	.	.	.	.	.	.
34	.	.	×	.	.	.	.	.	.	.
36	×	.	×	×	.	.	.	.	.	.
37	×	.	×	.	.	.	.	.	.	.
38	×	.	×	.	.	.	.	.	.	.
41	.	.	×	.	.	.	.	.	.	.
42	.	.	×	×	.	.	.	.	.	.
43	×	.	×	×	.	.	.	.	.	.
44	.	.	.	.	.	×	.	.	.	.
45	×	.	×	×	.	.	.	.	.	×

It is remarkable that one-third of the animals manifested signs of generalized vaccinia between the 12th and 13th day after immunization. Only lesions with virus isolation (CAM) were counted in Table VI.

As can be seen from Table VI, mostly the tongue, the lips, the throat and gingiva were affected by vaccinial lesions. The spontaneous development of vaccinial orchitis in two animals seems to be very remarkable. In one animal on the 5th day the left testicle was severely swollen and infiltrated. On the 8th day the right testicle was also involved. On the 11th day the redness and infiltration subsided. Histologically (Prof. Selberg/Hamburg) there was a marked oedema of the tunica propria, which was also visible in the big septal and interstitial parts. There was no cellular reaction and the canaliculi showed a completely normal structure.

Rabbits suffering from generalized vaccinia were obviously ill; they were sitting in a corner of the cage, refusing food or water. Therefore in all these animals loss of weight was explainable. Nevertheless it has to be stressed that the mortality of the animals affected from generalized vaccinia was not higher than that of the animals without generalization. Of 18 animals (out of 27) with generalized vaccinia 9 died, of the remaining animals 7 expired. Those animals surviving the crop of the vaccinial lesions showed healing of the pustules a few days later.

Table VII. *Comparison of different vaccinia strains in their capability to induce generalized vaccinia in asparaginase-treated rabbits*

Vaccinia strain	No. of animals	No. of gen. vaccinia	Fatal outcome	Asparaginase lot no.
Elstree	27	18	16	FB b 6366
EM 63	27	7	3	FB b 6366
EM 63	27	7	0	C 1783
Bern	27	1	2	C 1783
Hamburg	21	8	6	C 1783

(*b*) *Strain EM* 63 In a 2nd assay, 27 rabbits were vaccinated with the EM 63 strain and treated with A-nase (batch nos. C 1783, E 18 and E 21) analogously as in the first assay. In this trial only 7 animals showed generalized vaccinia (5 times on the eye and mouth, one in the intestine), no animal died.

In a subsequent trial 27 animals were vaccinated again with the strain EM 63, only the A-nase used was of another batch number (FB b 6366). Also in this assay, 7 animals showed a generalized vaccinia (4 times on the eye and genitals, twice on the stomach) and 3 animals died.

(*c*) *Strain Bern* In a 4th assay, 27 rabbits were vaccinated with the strain Bern, they got an i.p. A-nase treatment (batch no. C 1783) as above described. Only 1 rabbit developed generalized vaccinia (lesions on the mouth) and 2 animals died. Though the results were quite similar compared with the trial with strain EM 63, it has to be mentioned that the scab developed in general after vaccination with the EM 63 strain around the 5th day, whilst this occurred mostly 3–4 days later following immunization with the Bern strain. The dissolution of the scab followed the same schedule.

(*d*) *Strain Hamburg* From 21 rabbits vaccinated in the above manner with the strain Hamburg with additional i.p. A-nase treatment (lot C 1783: 18 E, 21 E, respectively), 8 animals demonstrated generalized vaccinia and 6 of them died. (Table VII).

CONTROLS

Two groups of seven rabbits were vaccinated with the Herzberg method using the strains Bern and EM 63 respectively. In these controls no generalization of the virus could be seen. All animals survived.

DISCUSSION

The immunosuppressive action of L-A-nase is known. Since the enzyme also inhibits the blastic transformation of lymphocytes (Astaldi), cells which are important mediators of the delayed type, it seemed feasible to induce generalized vaccinia by A-nase treatment in the animals. This was only possible after

hypocalcaemia, one serious side-effect of the enzyme treatment, had been overcome by calcium therapy. Tettenborn *et al.*(7) in a recent paper also found in rabbits treated with 1000–20000 u. of A-nase the same symptoms of hypoparathyroidism, independently from us. Evidently this stress reaction paved the way for the onset of generalized vaccinia which appears sometimes in humans, for example, after severe burns.

The simultaneous vaccination and enzyme treatment seems to be a model to study qualitative differences of some vaccinia strains, if the experimental conditions are similar. Where the frequency of cases of generalized vaccinia is concerned, the marked differences found between the strain Elstree and EM 63 are probably not by chance (Table VII). Unfortunately, the first-used batch of A-nase was not available from the producer for more trials. We assume that the later-used preparations were more purified. Perhaps it is an indication that the mortality of the vaccinated and enzyme-treated animals was greater in the first experiments (batch FB b 6366, Table VII) than in the subsequent studies. The later enzyme preparations also induced hypocalcaemia more rarely in our trials. Tettenborn *et al.* suggested that this effect might be due to a highly effective thermolabile contamination of the product. On the other hand, the frequency of generalized vaccinia was identical (see trial with EM 63 with the two batches, Table VII). Our results are in harmony with the study of Marennikova *et al.* (4). Also in this investigation with other methods the strain EM 63 showed the smallest capacity to multiply of all strains studied. This is a drawback to vaccine production since it is not easy to get reasonable titres. On the other hand, it is very remarkable that the strain EM 63, besides its low capacity to induce generalized vaccinia, had a better humoral antibody response (HI test) than the strain Bern. After immunization with the Herzberg method the geometric mean titre in 7 controls was 1:145 on the 19th day versus 1:113 in 6 additional A-nase-treated rabbits (Sindern, in preparation). The development of the vaccinial allergy was not inhibited in the EM 63-immunized and A-nase-treated animals (i.c. test with non-infectious smallpox vaccine on the 12th and 19th day of the trial). Therefore the EM 63 strain seems to us very suitable for the immunization of infants.

It is our opinion that our model gives us the opportunity to investigate some vaccinia strain characteristics.

SUMMARY

Rabbits treated with i.p. injections of 2000 u. of L-asparaginase 2 days apart develop in some cases clinical and chemical signs of hypoparathyroidism. This status can be overcome by calcium therapy. Among 27 animals treated this way and additionally vaccinated with the strain Elstree, 18 developed generalized vaccinia and 16 died. With the same procedure after vaccination with the EM 63 strain, only 7 animals showed a generalized vaccinia and 3 animals died. In similar experiments with the strains EM 63, Bern and Hamburg with a more purified lot of asparaginase, the strain EM 63 showed the lowest mortality. The development of the generalized vaccinia is explained by the inhibitory action of

the enzyme on the blastic transformation of the lymphocyte. The test may be useful to compare vaccinia strain characteristics and to have an insight into the pathogenesis of generalized vaccinia.

We are grateful to the Bayer Company, Leverkusen, Germany, for donation of 'Crasnitin', and to Dr K.-H. Meinicke, Krankenhaus St Georg, Hamburg, Germany, for the chemical blood analyses.

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Discussion

EDSALL (UK) How do you interrelate the hypoparathyroidism in your rabbits with the generalized vaccinia?

EHRENGUT (West Germany) There is no clear-cut causal relationship. It is possible that the stress reaction following the hypoparathyroidism in the rabbit paved the way for the development of generalized vaccinia. It is known in the human being that stress reactions like severe burns may provoke a generalized vaccinia until 3 weeks after vaccination.

NEFF (USA) Have you tried any challenge experiments on these rabbits?

EHRENGUT (West Germany) No, since we hardly expected to see any results from this.

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VACCINATION OF HEALTHY CHILDREN WITH CV1-78 AND CALF-LYMPH SMALLPOX VACCINE

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During 1972 the Committee on the Control of Infectious Diseases of the American Academy of Pediatrics and the US Public Health Service Advisory Committee on Immunization Practices recommended in the United States that it is no longer necessary to routinely immunize children against smallpox(**6, 7**). This recommendation was made in part because there are significant hazards associated with the current smallpox vaccine and in part because the incidence of smallpox is declining rapidly throughout the world.

In 1967 Dr Henry Kempe reported on the use of an attenuated vaccinia virus CV1-78 for vaccination of eczematous children to protect them against vaccinia(**3**). Subsequently the suggestion has been made that this strain might be used as an alternative to the current US vaccinia virus(**4**). I would like to present today some further studies of this vaccine, CV1-78, in normal children as compared to a group of normal controls who were vaccinated with the standard calf-lymph virus. This study was conducted at Johns Hopkins Medical School in 1970 in co-operation with the US Public Health Service Center for Disease Control. Dr Richard Wesley and Dr Wendell Speers were co-investigators in this study.

MATERIAL AND METHODS

CV1-78 vaccine was derived by Dr Thomas Rivers from the 1931 dermal vaccine virus of the New York Board of Health. It was subsequently propagated through four intratesticular passages in rabbits and then serially in chick embryonic tissue by Dr Parker and then Dr Kempe for a total of 78 passes. The material used in the study reported today was obtained from Wyeth Laboratories in lyophilized form along with the standard calf-lymph vaccine (Dryvax). Both vaccines had CAM titres between 10 log 7·7 and 7·9 pfu/per ml.

Vaccinations were performed with a bifurcated needle perpendicular to the skin. Five multiple punctures were used for primary vaccination and 15 multiple punctures for revaccination.

Clinical observations were made at the 7th postvaccinal day and serology was obtained at the 28th postvaccinal day.

Serological tests were performed at the Laboratories of the Communicable Disease Center. Neutralization antibodies were determined by the 50 % plaque reduction method of Cutchins *et al.* (**1**) and haemagglutination-inhibition (HI) antibody titres were measured by the Technique of Hierholzer, Suggs & Hall (**2**).

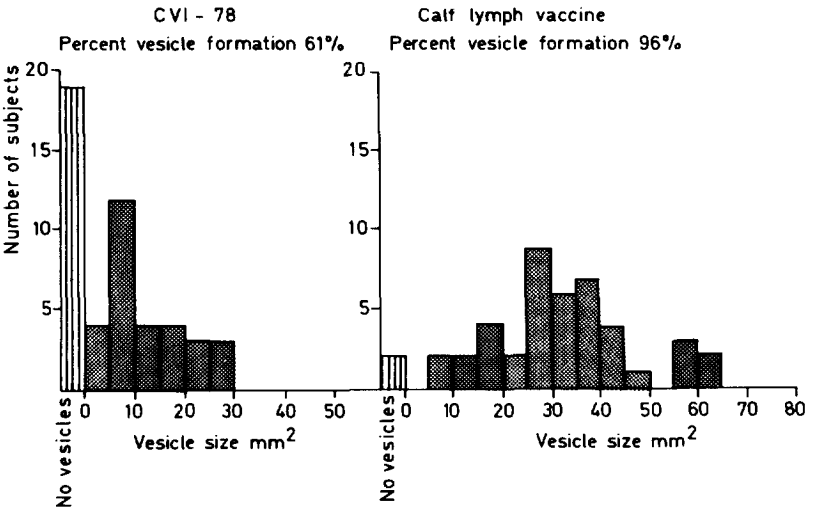

Fig. 1. Vesicle size at 7 days following primary vaccination.

Forty-nine normal children between the ages of 1 and 5 received primary vaccination with CV1-78 vaccine and 46 controls received primary vaccination with standard calf-lymph vaccine.

Twenty-six of the children who had received primary vaccination with CV1-78 and 22 of the children who received primary vaccination with standard calf lymph were available for revaccination with standard calf lymph 5–9 months later. All of these children were revaccinated with standard calf lymph that had a CAM titre of $10^{8.4}$ pfu/ml.

RESULTS

PRIMARY VACCINATIONS

Thirty of the 49 children vaccinated with CV1-78, 61 %, had a major reaction as measured by vesicle formation at the 7th post vaccinal day (Fig. 1). In contrast, 44 of the 46 children vaccinated with standard calf lymph, 96 %, developed a vesicle at 7 days. In general, the vesicles that developed following vaccination with CV1-78 were smaller than those that developed as a result of standard calf-lymph vaccination. Systematic reaction were also less in that only 8 % (4 of 49) of the CV1-78 subjects as compared to 26 % (12 of 46) of the calf-lymph subjects had a rectal temperature greater than 38 °C at the 7-day follow up.

Serologically there was very close correlation between HI titre conversion and the dermal take-rate. Sixty-seven percent of the CV1-78 subjects and 98 % of the calf-lymph subjects developed HI antibodies.

The rate of neutralizing antibody conversion, however, was surprisingly low in the CV1-78 subjects (Table I). Only 8 out of 49 (16 %) of the CV1-78 group developed postvaccinal neutralizing antibodies as compared to 41 out of 46 (89 %) of the calf-lymph group.

Finally, 13 children (27 %) who had received primary vaccination with CV1-78 had no postvaccinal dermal reaction, either vesicle or scar, and no postvaccinal

Table I. *Twenty-eight-day post-vaccination antibody results*

(Neutralization antibody result, ([10]log TCID 50/ml.)

Group	Post-vaccination – neutralizing titre range						
	<0·6	0·6–0·9	1·0–1·9	2·0–2·9	3·0–3·9	4·0–4·9	Total
CV1-78	41	2	6	0	0	0	49
CL	5	2	19	11	7	2	46

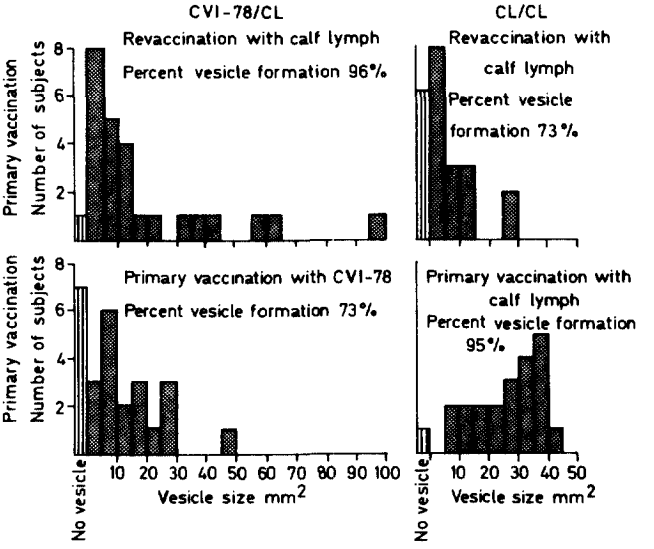

Fig. 2. Vesicle size following primary and revaccination.

circulating HI or neutralizing antibodies. In contrast, only one child (2 %) of those who received primary vaccination with calf lymph had no postvaccinal dermal or serological reaction. Thus, the highest possible dermal and/or serological take-rates was 73 % for the CV1-78 primary vaccinees and 98 % for the calf-lymph primary vaccinees.

REVACCINATION

From the original group the following were available for revaccination: 26 children who had received primary vaccination with CV1-78 vaccine and 22 children who had received primary vaccination with standard calf-lymph vaccine. Both groups were revaccinated with standard calf-lymph vaccine using 15 multiple punctures.

A major reaction was defined as evidence of vesiculation or eschar formation at the 7th day postvaccinal follow up (Fig. 2).

Table II. *Twenty-eight-day post-vaccination antibody result in CV1-78 and calf-lymph primary vaccinees revaccinated with standard calf lymph*

(Neutralizing antibody results, ^{10}log TCID 50/ml.)

Group	Post-vaccination – neutralizing titre range							
	<0·6	0·6–0·9	1·0–1·9	2·0–2·9	3·0–3·9	4·0–4·9	>5·0	Total
CV1-78/CL	7	2	5	2	2	1	1	20
CL/CL	0	0	3	5	5	3	3	19

Nineteen of 26 (73 %) CV1-78 subjects had had a primary take following the first vaccination. All but one (96 %) of the calf-lymph group had had a primary take following the first vaccination.

After revaccination all but one (96 %) of the CV1-78 group demonstrated a major reaction and 16 out of 22 (73 %) of the calf-lymph group had a major reaction. All subjects had a major reaction as a result of either the first or second vaccination. This revaccination response rate in 73 % of the calf-lymph group corresponds closely with the expected major reaction rate in recent, well-vaccinated subjects while the revaccination response in the CV1-78 group 98 % approximates that which would be expected in primary vaccinees or remote revaccinees(**5**).

Serologically, 100 % of both groups developed postvaccinal HI antibodies following either the first or second vaccination. The neutralizing antibody response, however, did not correspond with the HI antibodies response in the CV1-78 vaccinees (Table II). Thirty-five per cent of the CV1-78 group still had no neutralizing antibodies after revaccination with standard calf-lymph vaccine while 100 % of the control group did.

We may conclude from our study that CV1-78 *primary vaccination* when administered by the usual percutaneous route *results in a modified dermal and systemic reaction with persistent immunity in no more than* 80 % *of the subjects.*

Kempe *et al.*(**3**) have shown, however, that when the vaccine is administered by the subcutaneous route the take-rate approaches 100 %. This is now under further examination by a large comprehensive field trial under the auspices of the Vaccine Development Board. Van der Noordoa(**8**) has also shown that CVII – a strain which was also derived from Rivers 1931 dermal vaccine – had a 100 % take-rate. That strain is very similar to CV1-78 and perhaps it is the vaccination technique rather than the strains which accounts for the different take-rates between two vaccines.

Before closing and for the purpose of discussion I would like to speculate on the future of this vaccine. To begin with, the CV1-78 vaccine was presented originally to protect eczematous children from accidental exposure to vaccinia(**3**). Now that the practice of routine childhood immunization is no longer recommended in the United States and in many parts of the world, the chance that

an eczematous child will come in contact with the standard vaccinia virus hopefully will be diminished considerably, and therefore protection with another vaccine may not be necessary except in a very small select group. Secondly, vaccination with CV1-78 appears to be over-attenuated and alone may not offer sufficient protection against smallpox. Thus, primary vaccination with CV1-78 should be followed with revaccination with standard calf lymph. It is cumbersome to replace one procedure with two and there is no assurance that the combination of the two vaccines may not result ultimately in the same complication rate that would occur with vaccination with only standard calf lymph.

In conclusion, it is going to be difficult to replace an old vaccine with a new one. One returns to the impression that the best way to reduce the complications that result from smallpox vaccination is to continue to eradicate smallpox on a world-wide basis and reduce complications by reducing the number of vaccinations that would be required.

SUMMARY

Primary smallpox vaccinations were performed on healthy children comparing an attenuated chick embryonic tissue vaccine, CV1-78, to standard calf-lymph vaccine of a similar CAM titre, $10^{8.0}$ pfu/ml. Fifty-four children received primary vaccination with CV1-78 vaccine and 52 with standard calf lymph. CV1-78 vaccination resulted in a less severe clinical response and a lower percentage of dermal and serological conversion. Only 65 % of those vaccinated with CV1-78 developed a typical Jennerian vesicle as compared to 94 % with standard vaccine, and only 16 % of the CV1-78 vaccinees developed post-vaccination neutralizing antibodies as compared to 89 % of the calf-lymph vaccinees. Twenty-six of the CV1-78 vaccinees and 22 of the calf-lymph vaccinees received revaccination with standard calf-lymph vaccine 5–9 months after primary vaccination. Following revaccination, 96 % of the original CV1-78 vaccinees and 73 % of the calf-lymph vaccinees developed vesicular dermal reaction. Those who had failed to demonstrate either a dermal or a serological reaction to primary vaccination developed a typical primary-like Jennerian vesicle on revaccination. This was observed in 5 of the 26 CV1-78 subjects and 1 of the 22 standard calf-lymph subjects. Serologically following revaccination all children had positive HI titres, but only 65 % of the original CV1-78 vaccinees had positive neutralizing antibodies as compared to 100 % of the calf-lymph vaccinees. These results indicate that vaccination with CV1-78 vaccine does result in an attenuated response. Whether or not this vaccine is a reasonable alternative to standard calf-lymph vaccination is a matter for discussion and for further investigation.

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Discussion

HOCHSTEIN-MINTZEL (West Germany) Have you any explanation for the fact that some children who were prevaccinated with the attenuated strain and revaccinated with calf lymph did not develop neutralizing antibodies upon revaccination?

NEFF (USA) What I think happened is that in the CV1-group after inoculation virus replication is perhaps sufficient to produce cell-mediated immunity but not enough for stimulation of neutralizing antibodies. Then, when these children were challenged with the standard vaccine, the cell-mediated immunity inhibited the replication of the virus and, although there was another take, the amount of virus present was not enough to stimulate a neutralizing antibody response. It is therefore probable that the main protection is through cell-mediated immunity.

MARENNIKOVA (USSR) Was the potency of the tested vaccine equal to dermal vaccine and what was the titre?

NEFF (USA) The titre was similar. It varies between 7·6 and 7·9 pfu per ml.

ARITA (WHO) If you consider the individual response to vaccination with the CV-1 strain, many had a major reaction but failed to develop neutralizing antibody. How did a major reaction correlate with the development of HI-antibody?

NEFF (USA) In this study there is a very close correlation between dermal take and positive HI-titre. As I pointed out, there were a couple of cases that had no dermal take but did have a slight HI-titre and on revaccination they responded as revaccinees. But in general the HI-titres and the dermal reaction correlated very closely. There was really no correlation between the neutralizing antibody response and the dermal reaction. In other words there were quite a number that did not develop any neutralizing antibodies. There also is no way of telling about the size of the skin reaction, which individuals develop neutralizing antibodies and which do not. Does this answer your question?

ARITA (WHO) Yes, I think I understand that there is no correlation between the major reaction and the development of neutralizing antibodies.

NEFF (USA) Except for the fact that there were no individuals that had a positive neutralizing antibody reaction with negative dermal reaction and negative HI. This is the weakest measure of immunity within this particular study.

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PRELIMINARY RESULTS WITH THE HIGHLY ATTENUATED VACCINIA VIRUS 'MVA'

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Primary smallpox vaccination still implies a small but defined risk of complications. Experience has shown that such a risk is markedly lower in the case of revaccination. The object of prevaccination with attenuated vaccinia virus is to produce basic vaccinal immunity without the risk presented by conventional primary vaccination.

Previous trials with attenuated vaccinia viruses have been conducted with promising results, mainly by Rivers(4), Kempe(1), Noorda(3) and others. Actually the virulence of the attenuated vaccine did appear to be reduced generally, but as long as these vaccines still produce even a pustule, they can not really be considered as 'attenuated'.

'ATTENUATED' VACCINIA VIRUS STRAIN 'MVA' (MODIFIED VACCINIA VIRUS ANKARA)

Starting out from the investigations of a virus strain by Mayr & Munz(2) in Munich, the MVA strain has been developed to a very strongly 'attenuated' vaccinia virus. The original vaccinia virus was passed 523 times through cell culture passages. The virus plaques, visible on the chorioallantoic membrane (CMA) of embryonated eggs, are tender and small. They are confluent, depending on dilution. They can hardly be distinguished from variola virus when analysed macroscopically.

Animals tests have been conducted in Munich in collaboration with Dr V. Hochstein-Mintzel and Dr Huber and will be published in detail (1971, 1972).

Briefly, our results showed that the virulence and especially the neuropathogenicity of the MVA vaccinia virus strain are, as compared to the 'Elstree' vaccinia virus, much weaker.

White New Zealand rabbits infected i.v. with 10^5 pfu (plaque-forming units/CAM) of strain Elstree became severely ill, they remained almost without symptoms when infected with the same dose of the MVA strain; the titre of antibodies were, however, low (3/10 neg., 7/10 with HAH titre 1:16, NT 1:32). Only an intravenous MVA infection in a concentration of 10^6 pfu or higher regularly produced tangible clinical symptoms.

The intracutaneous injection of MVA (10^4–10^6 pfu) barely caused a skin infiltration with weak reddening on the fifth day p.i.

One group of 36 *baby mice* 2 days old (strain NMRI) received an intracerebral injection of 0·01 ml of a virus suspension of VV MVA corresponding to 10^5 pfu per dose. Another group received $7·5 \times 10^4$ pfu of VV Elstree. None of the mice treated with MVA died or even had tangible clinical symptoms, in spite of finding viable MVA virus in the brains of three of the mice 18 days after injection. All the animals injected intracerebrally with VV Elstree died between the 5th and 7th day after infection.

c 1. *Cynomolgus monkeys* (*Macaca irus*) were infected intracutaneously with 10^6 pfu of VV MVA in 0·2 ml suspension. There were no clinical symptoms postvaccination. Antibodies could not be found 21 days p.i. The challenge infection with Variola Major Virus, Surat strain, was executed on the 21st day p.i. with $1·4 \times 10^8$ pfu per animal i.v. One of the two unvaccinated control animals died 10 days p.i.; two other animals that had been pre-immunized with MVA did not become ill; four others contracted a light, modified course of variola between the 5th and 11th day p.i.

c 2. Five *Cynomolgus monkeys* were injected subcutaneously with 0·2 ml MVA. The challenge infection with Variola Virus (i.v., 10^7 pfu/animal), Madras strain, followed 2 weeks later. Three of the animals became slightly sick, two remained healthy. Three of the unvaccinated controls died and the other two became severely sick.

Further experiments involving monkeys and Variola Virus are under way.

No humoral antibodies could be found after the vaccination with MVA, but in spite of this a relative protection against variola has been established by the MVA inoculation.

Clinical *observations on humans* (ref. also Stickl & Hochstein-Mintzel(7) proved that the epicutaneous, conventional application of MVA is not successful, this vaccine must be injected.

The intracutaneous application in humans proved to be of advantage because the success of the vaccination is evident through a small, slightly swollen, easily perceptible red skin spot which appears on the 5th day p.i.

Humoral antibodies in humans were found only sporadically (3/18) and in low titres of neutralizing antibodies (1:4 and 1:8). The haemagglutination inhibition test against vaccinia virus was negative in all 68 tests conducted. General clinical symptoms were missing.

Over 1200 vaccine doses were sent out in 1971/2. Reports are starting to return and none indicates any undue side-effects nor any failure of the pre-vaccination with MVA. Independently, Goetz (1972) found the same results. Four hundred and two MVA vaccinations have been performed under our own control.

A small infiltrate or tiny hardened round red spot could be seen at the site of application 4–5 days p.i. About three weeks p.i. a small brownish spot with a tiny hardening of the skin could be seen. Between 5 days and 6 months p.i. conventional cutaneous vaccination was performed. All reactions appeared typically accelerated and modified. In rare cases, on the 5th to 7th day p.i., there was a pustular reaction accompanied by only a slight rise in temperature. No other general symptoms occurred.

In no instance has any exantheme or other hyperergic vaccination reaction been observed; the vaccination normally caused no general symptoms and no rise in temperature. It caused a small induration, which was covered by a scab on the 4th–5th day p.i. The scar was discrete.

Table I. *Vaccination with MVA*

	HIT	NT (14–30 days p.i.)	Skin + reaction
MVA	Neg. (68)	$\pm \begin{cases} 2 \times 1:4 \\ 1 \times 1:8 \end{cases} /14$	Intracut. inject.: tiny red spot from 5th to −20th day p.i.
MVA + convent. VV 'E'	1:16 (24)	1:32 + (20) (−1:64+)	Convent. vaccin. (after MVA): (*a*) 110/402 fever 1 × until 38 °C 16/402 fever 1 × until 38 °C (*b*) Accelerated revaccination reaction 402/402 (*c*) 28/402 pustule reaction with fever (*d*) 367/402 major reaction (r.r.) (*e*) 7 negative: no take

DISCUSSION

The MVA vaccination produced practically no humoral antibodies and after the subsequent conventional vaccination with the Elstree Vaccinia Virus the level of the humoral antibodies produced was lower than after primovaccinations without prevaccination with MVA (Table I). This low value also was evident after 3–5 weeks p.i. (MVA–VV) 1:16–1:32 in the haemagglutination inhibition test and 1:32–1:64 in the titre of neutralizing antibodies.

Summarizing, it can be stated that from 5 days to 6 months after the prevaccination with MVA, every primary vaccination with the Elstree strain developed identically to a normal re-vaccination.

The conventional primary vaccination of adults has such a mild course that the vaccinated persons did not have to be restricted in their normal activities nor did they ever complain of discomfort.

The amount of available data is not large enough for statistical indication of the risk incurred in the two-step vaccination against variola, using modified live vaccine for the prevaccination and subsequently applying the conventional material and method. From this point of view this report presents only first and preliminary results.

We may suppose that the MVA vaccine is able to create a basic vaccinal immunization against smallpox because of (1) the low virulence and the absence of neuropathological effects of the MVA as it has been demonstrated in the experiments conducted in animals, (2) the presently perfect record of the two-step primo-vaccination (MVA plus subsequent conventional smallpox vaccination) progressing exactly as a revaccination. It is to be expected that primovaccinations executed after an MVA prevaccination progress similarly to a revaccination, especially regarding postvaccinal complications which represent a strongly reduced risk in revaccinations. The prevaccination with MVA was

exclusively developed in combination with the conventional cutaneous smallpox vaccination.

SUMMARY

Primary smallpox vaccination is still accompanied by a small but defined risk of complications; such risk is markedly lower in the case of revaccination. The object of prevaccination with the highly attenuated live MVA vaccine is to produce basic vaccinal immunity without the risk of conventional primary vaccination.

Vaccinia virus was attenuated in 523 cell culture passages on chick embryo fibroblasts. The decrease in virulence was high. The local reaction after intracutaneous administration of the vaccine was mild; general symptoms did not occur. Subsequent epicutaneous vaccination with the conventional vaccine leads to typical revaccination reactions.

Experiments with animals had shown that the preceding immunization with MVA even protects Cynomolgus monkeys against a subsequent massive challenge infection with recently isolated variola virus (5×10^7 pfu). These preliminary tests conducted in combination with a highly attenuated vaccinia virus and subsequent conventional smallpox vaccination show promise of achieving primary smallpox vaccination with qualities of revaccination.

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Discussion

TAGAYA (Japan) You mentioned the absence of antibody in intradermally inoculated monkeys. Does this mean the absence of both HI-and neutralizing antibody?

STICKL (West Germany) Yes, we never had antibody after MVA.

TAGAYA (Japan) Have you inoculated monkeys intracerebrally with MVA.

STICKL (West Germany) Yes, we did: the dose was 10^6 pfu in 0·5 ml. Clinical signs of illness were not observed.

TAGAYA (Japan) Is your vaccine free of avian leucosis virus?

STICKL (West Germany) Yes, we use only leucosis-free eggs for the propagation of the virus.

HENDERSON (WHO) Dr Neff observed that one-third of the children in his study developed no neutralizing antibody following administration of the CV1-78 strain and subsequently the conventional strain. Did you also observe that some children did not develop any neutralizing antibody after the two injections?

STICKL (West Germany) Yes, we observed the same phenomenon.

EHRENGUT (West Germany) We have used the strain of Dr Mayr, Munich, since 1967 in eczematous patients or patients with special risks. I believe it is a good strain and a good procedure for complicated cases like eczematous patients. As to the mechanism of the mitigation of the local revaccination reaction, we have seen the development of vaccinial allergy. We do not suggest the use of this strain for general immunization and we are of the same opinion as outlined by Dr Neff regarding this subject. It seems doubtful whether vaccinial encephalitis can be prevented by this procedure as we are using a live vaccine.

STICKL (West Germany) We have never seen allergic reactions.

HENDERSON (WHO) I personally would be concerned about using either of the two vaccine regimes described by Drs Neff and Stickl in which some substantial proportion of children developed no neutralizing antibody. While there may be many different factors concerned in variola immunity, I find it difficult to be sanguine about potential immunity in the absence of neutralizing antibody. I wonder how the authors feel about this?

EHRENGUT (West Germany) Yes, we do not know about the role of neutralizing antibody in the prevention of smallpox. In our country smallpox is not endemic and what we are doing is vaccinating the people not against smallpox but against vaccination; it is a protection against the risk of the primary vaccination. In a case of contact with smallpox you always have to revaccinate. I think that if we get a riskless primary vaccination, it might be of use.

NEFF (USA) Dr Henderson, I would agree with what you have just said. But there is a small number of children with eczema, severe eczema, that may well go into areas where smallpox is endemic. With that group I would be willing to take the risk of vaccinating with an attenuated vaccinia virus first and follow up with the standard vaccinia virus. I do not know what this absence of neutralizing antibody means. You are just measuring one unknown risk against another. I would say that at least when you are dealing with a child with severe eczema the chances of his probably getting a severe eczema vaccinatum from vaccinia would be greater than the fact that perhaps the absence of these neutralizing antibodies means that he is not protected against variola. As far as I can see now, this is the only situation in which I will recommend the use of this two-step procedure of vaccination.

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THE RATIONALE FOR ELECTIVE PRE-VACCINATION WITH ATTENUATED VACCINIA (CV1-78) IN PREVENTING SOME VACCINATION COMPLICATIONS

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Over the past several years Wyeth Laboratories have distributed numerous batches of the CV1 vaccine for clinical evaluation of its attenuation in comparison to contemporary standard vaccine preparations. The inclusion in these trials of many subjects with eczema and other skin diseases initially tested the ability of this strain to avoid the complication of eczema vaccinatum to which these subjects are presumably at high risk. Then it became apparent that revaccination of the subjects in this clinical model could be carried out in reasonable safety, after appropriate interval, with a potent standard vaccine. If the attenuation of the CV1 strain is accompanied by a reduction in immunogenicity, then the two-stage procedure can theoretically add to the system the established capacity of the standard strain to offer protection against a possible challenge of variola virus.

This report condenses the available clinical data from the studies(1–19) shown in Table I into a general summary that affirms the validity of the proposed principle; namely that CV1 indeed actually protects against 'vaccination' and at least some of its complications. It is the revaccination with standard vaccine that presumably will protect against smallpox.

CLINICAL PROGRAMME

The investigations have accumulated more than 9000 immunizations with this strain, mostly in children 1–5 years old, but including also infants of less than 1 year and some older children and adults. Data on approximately 6000 control immunizations with standard vaccines at statutory titres are also included to provide a reactivity base-line.

The distribution of CV1 subjects by vaccination route is shown in Table II. Two main categories include about 5500 'normals' without evidence of skin disease and 3500 'eczemas', including all degrees of severity from a relatively few cases of acute, weeping infantile eczema through chronic or subacute cases, to those presenting only a history of the disease, but with clear skin at the time of vaccination. The latter include also a small percentage of subjects with allergies and skin conditions other than eczema that normally justify withholding regular vaccination in smallpox-free countries. Almost all of the recorded CV1 vaccinations were primary; about 5% were secondary or higher order. Vaccinations with the control vaccines were also primary. Approximately 60% of the CV1 doses were inoculated percutaneously with undiluted virus at titres ranging from

Table I. *Evaluation of CV1 strain – all reports (interim reports)*

Study	Investigator (ref.)	Approximate time	Subjects: Total no.	Subjects: Age
1	Kempe *et al.*(7)	Mar.–June 1966	594	Mostly 1–5 years
2	Kempe *et al.*(8)	Fall 1966 to Mar. 1967	1009	3 months to 65 years; mostly 1–5 years
3	Kempe *et al.*(0)	Fall 1967 to spring 1969	2451	
4	Kempe *et al.*(0)	Fall 1969 to present time	309	240, 1–5 years
5	Sedlis(13)	Spring 1967 to summer 1969	201	.
6	Sedlis(14)	Aug. 1969 to Aug. 1970	71	.
7	Sedlis(15)	Sept. 1970 to 1971	58	1–14 years
8	Benenson(3)	1969–1970	16	11, 1–6 years; 5, 10–27 years.
9	Arthur(1)	Nov. 1967 to June 1969	234	Infants and children
10	Arthur(2)	July 1968 to Mar. 1970	80	Infants and children
11	Hirayama(5)	Spring–Summer 1969	91	
12	Kimura(9)	1969	63	18, 5–7 months; 34, 3–6 years
13	Kawana(6)	Fall 1968	56	3 months to 2 years
[14	Tagaya *et al.*(16)	1970/1971	1566]	.
15	Tagaya *et al.*(17)	1970/1971	4511	.
16	Minimitani *et al.*(11)	Spring 1968 to spring 1969	413	Primary, 358 infants; revax, 55, 6–12 years
17	Minimitani *et al.*(12)	Fall 1970 to spring 1971	340	28, 6 months to 1 year; 155, 1–2 years; 157, 2–12 years
18	Wesley *et al.*(19)	Spring 1970	54	1–5 years
19	Ducksburg *et al.*(4)	Oct.–Dec. 1970	82	1–3 years
	Total subjects (excluding interim reports)		9030	

$10^{7.2}$ to $10^{8.4}$ CAM pock-forming units at the time of vaccination, usually applied by three to seven multiple pressures or punctures with a bifurcated needle. In some studies the vaccine was also diluted for subcutaneous or intracutaneous administration at titres ranging from about 3·5–5·5 logs.

Local vaccination responses were assessed by various methods but generally classified in relation to standard vaccine reactions. Some (**1, 2, 7, 8, 18**) used an arbitrary classification from zero to 4+, with a 'no-take' reflecting absence of any skin necrosis or defined as equivocal by WHO standards; a 3+ or higher response resembled a typical 'major' response following standard vaccine. Others scored generally by lesion parameters, including vesicle and erythema areas or diameters. For subcutaneous responses, where a

Table II. *CV1 vaccine – distribution* of subjects by route†*

		Primary			Revaccination		
			Sub Q			Sub Q	
Skin condition	Subjects	MP	HI	Low	MP	HI	Low
Eczema	3446	1467	306	1370	90	33	180
Normal	5512	3449	841	1048	44	53	77
Total	8958	4916	1147	2418	134	86	257

* Figures exclude interim reports.

† Routes:

MP, percutaneous, $10^{7\cdot2-8\cdot4}$ pfu/dose.

SQ, HI, subcutaneous and intracutaneous, $10^{4\cdot2-5\cdot8}$ pfu/dose.

SQ, Low, subcutaneous, $10^{3\cdot2-4\cdot4}$ pfu/dose.

central vesicle and/or necrosis was absent, generally the measurements included local erythema, swelling and/or induration diameters.

Examination for systemic reactions and complications was usually made by the parent or investigator 7–10 days after vaccination, but sometimes for periods of 2 weeks or longer, and individual fevers, malaise, lymphadenopathies and other systemic responses were recorded. Serological responses to CV1 vaccination were documented for almost 1000 percutaneous vaccinees, and more than 800 subjects immunized subcutaneously, and rises in either serum neutralizing (**7, 8, 19**) or haemagglutination-inhibiting (**4, 5, 6, 11, 12, 16, 17, 19**) antibodies were reported, or their respective geometric mean titres were calculated for comparison to comparable control responses.

Percutaneous revaccination of approximately 4500 CV1 primary vaccinees, including more than 1100 eczemas, was carried out with the corresponding standards employed in the respective countries. Local and systemic responses were recorded by the same criteria employed in the primary studies. Intervals from primary to revaccination were quite variable, ranging from 1 to 12 months, but most were in the 1- to 6-month period.

TRIAL RESULTS

Table III summarizes the *severity of local responses* following percutaneous vaccination for approximately 4400 CV1 doses, as compared to 6000 subjects vaccinated with the corresponding standards. All responses clearly defined the comparative attenuation of the CV1 strain. On average, more than 94% were 'milder', or more 'moderate', than the usual standard 'major' response, and 27% overall were 'equivocal' or 'no-take'. The standards on the other hand had only a 3% no-take response, with 88% in the major category. There was a small component of milder responses to the Lister strain control used in the 1970/1 Japanese programme and reported for about 500 subjects (**17**), but this sample was clearly less reactive than the other controls, including other preparations of the Lister strain which otherwise responded with 94–100% major takes. The general take-rate for the CV1 strain was not significantly related to skin condition, nor was there a significant correlation between the severity of

Table III. *Local responses to primary vaccination – percutaneous*

Vaccine	Skin condition*	Studies included	Subjects	Responses				
				Severity < standard				Major ⩾ standard
				No take	1+ or 2+	Mild†	Moderate‡	
CV1	E	3, 7, 8, 11, 12, 15, 19	1054	237	104	41	652	20
	E+N	4, 6, 17	452	97	.	103	.	252
	N	9, 10, 11, 12, 13, 15, 16, 18, 19	2918	862	99	160	1797	.
CV1 total			4424	1196 (27 %)	203 (5 %)	304 (7 %)	2449 (55) %	272 (6 %)
Ikeda	N	11, 14	1550	13	.	.	.	1537
Lister	N§	11, 14, 15, 19	2559	133	.	521	.	1905
Equador	N	11	1846	47	.	.	.	1799
CLV-NYC	N	18	52	3	.	.	.	49
Controls total			6007	196 (3 %)	.	521 (9 %)	.	5290 (88 %)

* E, eczema; N, normal skin.

† Mild response: central vesicle 2–5 mm diam. and/or erythema 4–12 mm diam.

‡ Diameter of vesicle *or* erythema less than standard vaccine.

§ Includes 145 subjects with eczema and other skin diseases.

Table IV. *Maximum local reactions – primary vaccination: percutaneous*

Vaccine	Skin	Study report	Subjects measured	Days	Mean diameters (mm)		
					Vesicle	Erythema	Induration or swelling
CVI	Eczema	11	12	10	3·7	16·3	12·5
CVI	Normal	11	49	10	3·0	10·9	8·3
Ikeda (A)	Normal	11	44	10	8·6	41·1	27·7
Ikeda (B)	Normal	11, 14	1493	8	7·5	22·9	18·2
Lister (A)	Normal	11, 14	1880	8	6·8	17·6	15·3
Equador	Normal	11	1799	8	.	19·2	17·4
CVI	Eczema	14	4	7	2·6	6·2	3·0
CVI	Normal	14	36	7	3·1	7·8	3·7
		.	44	9	4·2	18·3	11·4
CVI	Eczema	15	138	7	3·5	8·1	7·3
		.	59	9	5·5	20·9	15·2
CVI	Normal	15	748	7	3·6	8·3	6·8
		.	166	9	5·7	21·1	17·2
Lister (B)	Eczema	15	25	7	4·7	11·7	9·5
Lister (B)	Normal	15	140	7	5·4	12·1	9·9
CVI	Eczema	12	11	.	2·5	7·2	6·3
CVI	Normal	12	27	.	4·2	10·5	6·2
CVI	Normal	16	53	.	< 5	95 % < 5, 5 % 5 to < 15	.
CVI	Norm. + Ecz.	17	103	.	< 5	< 10	< 10
CVI	Normal	18	54	7	2·8–3·2	.	5·9–6·1*
CLV-NYC	Normal	18	52	7	5·7–5·8	.	10·6–10·8†
CVI	Normal	19	61	12	5·6	19·1	.
CVI	Eczema	19	21	12	6·2	27·0	.
Lister (C)	Normal	19	26	7	10·3	19·2	.

* Scar: 4·7 mm.
† Scar: 6·8–6·9 mm.

Table V. *Maximum local reactions – primary vaccination: subcutaneous*

Vaccine	Skin	Study report	Subjects measured	Days	Mean diameters (mm) Erythema	Mean diameters (mm) Induration or swelling
			High dose ($10^{4\cdot2-5\cdot8}$ pfu)			
CV1	Normal	14	113	7	13·8	12·3
			165	9	23·0	15·3
CV1	Eczema	14	11	7	17·5	12·7
			7	9	20·7	13·7
CV1	Normal*	16	41	8	<5	5–20
			15	10–16	5–10	10–30
			Low dose ($10^{3\cdot2-4\cdot4}$ pfu)			
CV1	Normal	14	43	9	19·6	15·3

* Includes intracutaneous dosage.

local response and the precise amount of virus in the range from $10^{7\cdot2}$ to $10^{8\cdot4}$ CAM/pfu per vaccine dose administered percutaneously.

'No-takes' were considerably higher for all vaccine dilutions administered subcutaneously, varying between 50% and 75%, depending upon dose. The local effects were mostly 1+ or 2+ (**7, 8, 18**), with only a rare 3+, or else the areas of reactivity were measurably less than the standard (**11, 12, 16, 17**).

The physical dimensions of the maximum local reactions following CV1 primary, percutaneous vaccination were generally smaller than the standard (Table IV). Although lesion size varied with day of observation of a 7- to 10-day period, central vesicles ran about half smaller with CV1, and erythema about a third to a half of the corresponding control dimensions although the milder Lister standard earlier noted in the Japanese trial (**17**) yielded significantly reduced erythemas. Induration or swelling was 8–17 mm versus 10–28 mm in controls. Two studies, 15 and 19, reported a trend to somewhat delayed and larger erythemas with extended observation of CV1, and study 19 also suggested this tendency increased in a comparatively small group of eczematous subjects.

CV1 lesion scores of major responses by subcutaneous vaccine, as seen in Table V, tended to equate with the larger dimensions of erythema and induration of the corresponding percutaneous scores.

Systemic reactions following primary percutaneous vaccination with CV1 were clearly also milder than with the standard strain. The American studies (**1–3, 7, 8, 10, 13–15, 18, 19**) on almost 1000 vaccinees observed for periods up to about 10 days showed comparatively rare fevers exceeding 103 °F. About 5–10% were in the range of 101–103 °F and generally 90–95% were less than 101 °F. Corresponding responses from the calf-lymph control strain were 35% fevers at 101–103 °F and 5–10% exceeding 103 °F. Other systemic effects of

Table VI. *Serological responses of primary vaccinees: percutaneous vaccination*

Study	Vaccine	Skin	Antibody	Time (months)	Local reaction	No.	%	G.M. titre
1	CV1	E	Neut.	1	.	101	'All'	52·1
	CLV-NYC	N	Neut.	1	.	36	'All'	48·5
2	CV1	E	Neut.	1	.	162	—	40·3
	CLV-NYC	N	Neut.	1	.	43	—	41·5
15	CV1	E+N	HI 'rises'	1	+	120	95	12·6
					−	38	74	10·2
				2	+	40	100	12·3
					−	9	89	9·3
16	CV1	N	HI ⩾ 1:4	1	+	25	89	11·7
					−	7	57	4·0
17	CV1	E+N	HI 'rises'	2	.	53	96	18·8
				4	.	23	87	18·1
				6	.	37	31	4·7

CV1 accompanying fever were about 15% malaise of mostly 1+ severity and only about 1% with illness comparable to the standard primary reaction. About the same magnitude of axillary lymphadenopathies were noted, and in the entire series there were only three cases of erythema multiforme reported and several maculopapular or erythematous exanthemas. The Japanese studies(**5**, **16**, **17**) reported fever in fewer than 2–5% of more than 3000 CV1 vaccinees after 7–10 days, increasing to about 10% by the fourteenth day. In contrast, data for standards in the Japanese control programme ranged from 21% to 29% fever occurrence. The milder Lister control used in the 1970/1 programme yielded fever in 4–5% and in 12% of the subjects after 7 and 14 days respectively.

The overall systemic reaction following subcutaneous vaccination generally paralleled the percutaneous responses to CV1.

Post-percutaneous vaccination serological responses showed a fairly high order of antigenicity in terms of some antibody rises, or their geometric mean titres, notwithstanding the comparatively high rate of non-takes already noted, especially for vaccine given by the subcutaneous route. Generally, however, the mean titres were lower than those obtained with the corresponding control vaccines. Study reports 1 and 2 from Dr Kempe (Table VI) observed development of neutralizing antibody with CV1 in almost all eczematous subjects, matching the calf-lymph control with normals at titres from 1–40 to 1–50. On the other hand, Dr Neff(**19**) earlier reported induction of only 16% SN antibodies in contrast to 89% for the standard, with about a sevenfold higher mean titre in the latter. On the other hand, HI antibody rises to CV1 were fairly high overall, ranging from 70% to 100% and matching control levels. Mean titres

Table VII. *Serological responses of primary vaccinees – 1 month postpercutaneous (MP) vaccination*

Study	Vaccine	Skin	Subjects	Antibody	Response		Remarks
					%	G.M. titre	
11	CV1	E+N	40	HI ≥ 1:4	90 (78 % ≥ 1:8)	12·9	Major responders only
11, 14, 17	Ikeda (B)	N	74	HI ≥ 1:4	98 (95 % ≥ 1:8)	19·5	
	Lister (A)	N	105	HI ≥ 1:4	82	24·0	
	Equador	N	208	HI ≥ 1:4	98	18·2	
13	CV1	N	56	HI ≥ 1:8	70 (39/56)		Random sample with only 31 'Majors'
14	CV1	E+N	115	HI ≥ 1:8	90	19·5	Random sample of infant population
18	CV1	N	49	HI ≥ 1:8	67	11·4	All subjects
				Neut. ≥ 1:4	16	19·0	Skin reactors only
	CLV-NYC	N	46	HI ≥ 1:8	98	20·5	All subjects
				Neut. = 1:4	89	148·0	Skin reactors only
19	CV1	N	52	HI ≥ 1:10	100	56·6	Responses unrelated to severity of local and systemic reactions
	CV1	E	18	HI ≥ 1:10	94	67·9	
	Lister (C)	N	9	HI ≥ 1:10	100	92·5	

Table VIII. *Serological responses of primary vaccinees – subcutaneous vaccination*

Study	Vaccine	Skin	Antibody	Time (months)	Local reaction	Vaccination route† S.Q. ($10^{4\cdot2-5\cdot4}$ pfu) No.	%	G.M. titre	S.Q. ($10^{3\cdot2-4\cdot4}$ pfu) No.	%	G.M. titre
1	CV1	E	Neut.	1	.	54	'All'	54·4	60	'All'	*c.* 30
	CLV-NYC	N	Neut.	1	.						
2	CV1	E	Neut.	1	.	112	.	48–63	113	.	24–37
	CLV-NYC	N	Neut.	1	.						
15	CV1	E+N	HI 'rises'	1	+	.	.	.	8	100	14·1
					−	.	.	.	78	91	11·1
				2	+	9	100	10·8	15	100	12·3
					−	25	92	11·1	74	97	12·9
						S.Q. ($10^{4\cdot63}$ pfu)			I.Q. ($10^{4\cdot63}$ pfu)		
16	CV1	N	HI ⩾ 1:4	1	+	16	94	7·3–11·7	37	92	7·9
					−	30	100	8·1	64	94	7·2
						S.Q. ($10^{5\cdot6-5\cdot8}$ pfu)			S.Q. ($10^{4\cdot6-4\cdot8}$ pfu)		
17	CV1	E+N	HI 'rises'	2	.	45	73	16·7	36	92	20·1
				4	.	60	82	13·4	39	79	17·5
				6	.						

again, however, were less than the corresponding averages with the standard vaccine.

The *relationship to control HI responses* may be noted in Table VII, where the control data in terms of conversion percentages and mean titres are shown. Dr Neff's data are shown for study 18. It is certainly evident from these data that sero-conversions can take place in subjects not experiencing any local or systemic responses, particularly when measured by the HI response. This is especially true for diluted vaccine administered subcutaneously; as noted earlier 'take' failures were quite high, but sero-conversions, as measured by either antibody, attained levels in some instances matching the multiple-pressure experience. Table VIII shows these responses – the high percentage conversions are evident.

Percutaneous revaccination of CV1 primary vaccinees with potent standard vaccine strains showed widely varying 'major' response frequencies, but local and systemic reactions were mostly modified, and considerably milder than usually seen with the standard. In fact, overall, the revaccination reactions were comparable or even milder than those originally following CV1 vaccination. Generally, the incidence of major reactions did not correlate with specific time intervals(**17**). In two reports(**9, 15**), where CV1 was used as a revaccinating strain, additional 'takes' were produced.

Generally, the induction of a major response on revaccination is assumed to compensate for an earlier vaccination failure with CV1, but evidently the procedure can nevertheless induce measurable antibody and Dr Neff(**19**) has shown that revaccination with the standard strain is clearly able to increase significantly the serum neutralizing antibody deficiency of the CV1 treatment: a pre-vaccination level of about 10% was raised to about 65%; but, pre- and post-HI-antibody levels were approximately equally high after the two procedures.

DISCUSSION

A useful substitute for current standard vaccination must afford equal protection against smallpox, but the strain should be less 'virulent' in terms of producing fewer gross local and systemic responses that must necessarily lead to the complications of routine vaccination. Study of the CV1 strain in thousands of subjects has established its attenuation by such criteria, supporting its eligibility as a potential substitute; but of course its capability of protecting against variola is essentially unknown. However, by the definition of attenuation, a test of this potential is no longer possible in terms of conventional skin reactions alone; nor is it possible ethically to establish a direct test of protection against variola.

The present clinical programme has taken an alternative route in demonstrating the ability of the CV1 strain to protect against *vaccine* virus in preventing the complication of eczema vaccinatum, and its effectiveness to this end seems equally as clear as its attenuation for human skin. The best estimates of the frequency of this complication project that between 25 and 50 cases might have occurred among the more than 3500 'eczemas' receiving the CV1 strain as a

primary vaccine; but in fact none did occur. Furthermore, revaccination of a large part of this population, including 1100 'eczemas', with standard vaccines again saw the absence of this complication.

The data are thus consistent with the thesis that preliminary vaccination with the CV1-78 strain can reduce a number of the complications of later vaccination with a standard strain, and the combined procedure can probably result in the same overall 'takes' level as primary standard vaccination alone. A comparable level of circulating HI-antibody will probably be induced, but perhaps a lesser percentage of neutralizing antibody, although a failure in some cases to match neutralizing antibody levels with the generally high serological conversions by HI antibody, with or without a 'major' reaction, may only reflect the controlling relationship of complement in the former determination. In either case, the severe skin reaction of unprotected standard vaccination is significantly avoided. Presumably enough antigenic stimulus is applied in the CV1 pretreatment to afford at least a dermal or cellular immunity which is then capable of reducing virus replication on standard virus challenge. The combined process, however, compensates to some degree for the deficiencies in raising antibody that may accompany the attenuation of the CV1 strain.

SUMMARY AND CONCLUSION

In extensive clinical studies, 5500 subjects with normal skin and 3500 with eczema and other skin diseases received primary vaccination with the attenuated CV1 vaccinia virus with significantly reduced local and systemic responses. Presumably sufficient antigenic stimulus was imparted to allow later revaccination of these subjects with standard, more virulent, vaccine strains without the severity of the corresponding reactions usually accompanying standard vaccination. The two-stage procedure appeared to have eliminated an expected incidence of eczema vaccinatum in the large population at risk of this complication. Confirmation of CV1 attenuation in this model suggests that prevaccination of eczematous children with this strain can offer protection against elective or contact exposure to standard vaccine; and furthermore, the combined procedure may indeed offer a substitute for conventional vaccination with its higher morbidity potential.

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Discussion

TAGAYA (Japan) We have been studying the CV-1 strain in Japan. Revaccination was done with the Lister vaccine. The reaction with the CV-1 strain came somewhat later than with the Lister vaccine but the take-rate of the CV-1 strain was higher. The lower take-rate with the Lister vaccine may be accounted for by the milder pressure with the bifurcated needles when administering the Lister vaccine than when they gave the CV-1 strain. Those showing a reaction with the CV-1 strain were strongly immunized but of those with an artificial reaction half failed to react to the Lister vaccine. Subcutaneous inoculation appeared to give a little less immunity.

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ATTENUATED VACCINIA STRAIN FOR PRIMARY VACCINATION OF ADULTS IN NON-ENDEMIC COUNTRIES

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In this paper the use of the Rivers CV II strain is discussed. Most of the facts have been published before(2), but repetition may be useful.

The idea behind it was initiated by Lt.-Col. Beunders. He was responsible for the state of health of the Dutch Army, and as he had to perform, for reasons already exposed by Polak, a large number of primary vaccinations in recruits, he was worried by the amount of vaccination illness. He was thus one of the initiators of the comparative vaccine trial which resulted in the general preference for the Elstree strain(1). Furthermore he wanted to experiment with a harmless prevaccination procedure, and in spite of the fact that he had committed himself to a study of inactivated vaccinia, he stimulated and made possible the work with attenuated live vaccinia. Those concerned with this work, including the speaker, should stand back a little and give him all due credit.

CV II was chosen because it seemed, from Rivers' published work, such a very poor vaccine, ideal for prevaccination. Very soon it was obvious that this was due to the low titre (10^4) in the chick embryo tissue culture system used by him and, if prepared on the chorioallantoic membrane, titres around 10^8 were easily obtained. Application on the skin of this material gave quite different results: more than 95% takes on primary vaccination and good antibody response. More than 60000 recruits have been vaccinated with this strain.

If only skin reactions on revaccination are taken as a measure of immunity, the resultant immunity after CV II would be comparable to that after calf lymph – but neutralizing antibody titres (measured by the 50% plaque reduction method) are $\frac{1}{2}$–$\frac{1}{4}$ those after vaccination with calf lymph. Evidently, the lesser amount of viral antigen produced has consequences only for humoral, not for cellular immunity (Fig. 1, Table I).

We have recently traced three people who received primary vaccination with the CV II strain, two of them 7 years ago and the third 10 years ago without subsequent revaccination. After 7 years one had a neutralization titre (50% plaque reduction) of 8, the other of 90. The individual with the lowest antibody titre has been revaccinated with the Elstree strain, resulting in a small accelerated reaction. In the second candidate for revaccination the changes in cellular immunity on revaccination will be studied *in vitro*. The third (10 years) has been

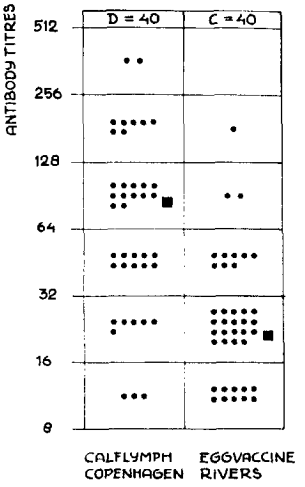

Fig. 1. Neutralizing antibody titres in serum samples 1 year after primary vaccination with calf lymph (Copenhagen strain) and egg vaccine (Rivers strain). Two groups of 40 soldiers each. ●, Individual titre; ■, median titre.

Table I. *Skin reactions following revaccination with calf lymph*

Revaccination	Total no. of men	Type of skin reaction: Accelerated reactions: Severe	Inter-mediate	Mild	Imme-diate reaction	Acceler-ated reaction (%)	Imme-diate reaction (%)
Copenhagen vaccine 3 months after egg vaccine	43	—	3	14	26	40	60
Copenhagen vaccine 6–9 months after egg vaccine	47	1	26	13	5	90	10
Elstree vaccine 6–9 months after egg vaccine	56	—	20	18	18	70	30
Elstree vaccine 12 months after egg vaccine	40	1	25	8	6	85	15
Elstree vaccine 12 months after Copenhagen vaccine	30	1	12	11	6	80	20

Table II. *Calculation of 'index of pathogenicity' for various vaccines (modified from table* X *of Polak & Beunders,* 1963)

Vaccine	Days of fever > 38·7 °C / Vaccinations with various vaccines	Days of fever > 38·7 °C / Vaccinations* in control groups with Copenhagen strain	Index of pathogenicity†
Copenhagen	276/529	276/529	100
München	34/93	42/108	94
Ecuador	24/107	30/78	58
Ecuador-Nih‡	22/75	34/63	54
Elstree	42/285	170/270	23
Elstree-Nih‡	32/218	99/191	28
Rivers	93/4000	'651/1239'	4·4

* From same trial groups as second column except the Rivers control group.

† Index of pathogenicity $= \frac{\text{rate of second column}}{\text{rate of third column}} \times 100$.

‡ Prepared at the National Institute of Health from Ecuador vaccine through three calf passages.

Table III

Vaccine strain	Elstree	Rivers
Number of soldiers	92	99
Days at 38 °C	109	45

revaccinated with the Elstree strain (no antibody titres available). Both revaccinations provoked accelerated reactions, with scab formation on day 7.

I mention these cases only as an illustration of our conception that cellular immunity after vaccination with the Rivers strain is probably not very different from cellular immunity after the use of calf lymph.

Table II shows the index of pathogenicity calculated against the trial of Polak and Beunders. It may be objected that these trials were not under identical conditions as they were done in different years. We did, however, conduct a comparative trial in the same barracks at the same time, necessarily on a smaller scale (Table III).

It is evident that the Rivers CV II strain has a lower pathogenicity than the Elstree strain.

As this is a prevaccination – when does one revaccinate? If revaccination is done within 2 weeks of the primary vaccination there is no booster response in antibodies, if revaccination is done after the third week we obtain a definite booster response resulting in higher titres of neutralizing antibody than after primary vaccination with the Elstree strain alone. For this reason we prefer

not to revaccinate earlier than the 3rd week, and, considering that cellular immunity and skin reactivity do not differ significantly from those after primary vaccination with calf lymph, there is no time limit, but we instruct our vaccinees not to postpone the final vaccination more than 3 years.

Which are the indications for use of this vaccine? Almost exclusively primary vaccination of adults of the following groups:

(*a*) medical and paramedical professions in non-endemic countries;

(*b*) military personnel in non-endemic countries;

(*c*) overseas travellers (adults and children).

Group (*c*) should re revaccinated after 3–4 weeks, groups (*a*) and (*b*) within 3 years. The Rivers vaccine is also used by us for revaccination (if the primary vaccination was performed more than 10 years ago) in 'high-risk' individuals: patients with eczema, pregnancy, neurological disorders – who must be vaccinated for overseas travel.

In the future, with the sensible (and long overdue) abolition of general infant vaccination and the disappearance of the smallpox scare (even with sporadic persistence of smallpox foci), groups (*a*) and (*b*) will continue for several decades to need a basic immunity and this can be provided by an attenuated vaccine as it is now provided in the non-endemic countries by a useless infant vaccination. As different vaccination strategies are necessary in endemic and non-endemic countries, so different vaccines should be used to attain different aims in different situations. A price in vaccination illness and discomfort which is permissible in the presence of a constant threat of smallpox is no longer so if the risks are minimal.

For the past 10 years we have made extensive use in several Amsterdam hospitals of the Rivers strain (of which the seed virus has been freed of avian leucosis viruses) to vaccinate doctors, medical students, nurses and personnel in general. The negligible illness and loss of working days are greatly appreciated by the vaccinees, but most of all by the hospital management: their former resistance to vaccination has disappeared.

This conference marks the end of centuries of irrational smallpox scares and consequently irrational ways of combating this terrible disease. I feel that now more attention should be given to better vaccines of minimal pathogenicity and consequent easy acceptance. The old historical strain of Rivers might fill this need until new strains are developed with more sophisticated modern virological techniques.

SUMMARY

With the decreasing incidence of smallpox and increasing insight of public health authorities it may be possible to abolish infant vaccination in all non-endemic countries and perform the necessary primary vaccination of adults with an attenuated vaccine. The properties of Rivers' CV II strain and its extensive use in The Netherlands for this purpose will be discussed.

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Discussion

GISPEN (The Netherlands) I am informed that Rivers' vaccine has been used in the Dutch Army in several ten thousands of people. Has Dr Dekking seen the results in terms of complications in the central nervous system? If so, can he give us some figures of the frequency of such reactions?

DEKKING (The Netherlands) There was one certain case of encephalopathy, a mild one, and there were two dubious ones in more than 65 000 vaccinees.

GISPEN (The Netherlands) Is Dr Dekking aware of the fact that this frequency is about the same as that after use of the Dutch smallpox vaccine prepared as calf lymph from Elstree virus?

DEKKING (The Netherlands) With such low frequencies at least $2\frac{1}{2} \times 10^6$ adults should receive primary vaccination with both strains to allow a valid comparison. Furthermore, the CV II strain is preferable, not because it might cause less-frequent central nervous system complications (no reliable estimate can be made of these) but because it evidently causes a less severe vaccination illness and for that reason may be more acceptable.

EHRENGUT (West Germany) You suggested using Rivers' strain when the smallpox eradication programme succeeds in the near future but you took hospital personnel as a group to be vaccinated with this strain after abolishing mandatory vaccination. I feel this is the only group where we should not use such attenuated strains since we know that 69 % of all variola cases in Germany since 1947 arose in the hospitals.

DEKKING (The Netherlands) Could you give figures about the percentage of hospital personnel in Germany that has been vaccinated? I think if my recollections are right that many of the victims of smallpox in German hospitals had never been vaccinated. The problem in hospital vaccinations is that the choice is not between hospital personnel vaccinated with calf lymph or with CV II but between personnel not vaccinated at all (because of mental resistance) or personnel which have at least received a basic immunity.

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PROPERTIES OF AN ATTENUATED MUTANT OF VACCINIA VIRUS, STRAIN DIs

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A mutant of the Dairen-I (DI) strain of vaccinia virus was obtained during serial passages of the virus through 1-day eggs. The mutant was characterized by the formation of smaller pocks on the chorioallantoic membrane (CAM) or embryonated hen's eggs than those formed by the parent strain, and was almost avirulent in experimental animals other than chick embryos. The mutant produced no cytopathic effect (CPE) in most of the cell cultures examined except chick embryo cell cultures (CE)(**13**). The mutant produced, however, mild skin lesions when inoculated to cynomolgus monkeys by scarification of the skin or intradermally, inducing the production of haemagglutination inhibition (HI) and neutralizing antibodies(**5**). Properties of the mutant were further studied to examine possible availability of the mutant as smallpox vaccine.

RESULTS

HOST RANGE OF THE DIS VIRUS IN CULTURED CELLS

As reported in a previous publication(**13**), the DIs virus produced no CPE in HeLa, FL, L and primary monkey kidney (MK) or rabbit kidney (RK) cell cultures, although equivocal growth of virus was suggested. Table I shows a summarized data of the susceptibility of various cell cultures to the DIs virus. Except CE, primary human embryo kidney (HEK) and guinea-pig kidney (GPK) cell cultures only supported the growth of virus, although the yield of infective virus was low. The results of comparative titrations of the mutant and parent virus are shown in Table II. Plaque assay in CE monolayers was considered as a method of choice for titration of the DIs virus. In this connexion it was also found that the incorporation of DEAE-dextran from 200 to 400 μg/ml in agar overlay improved the efficiency of plaquing of the DIs virus about 2 times both in size and number. The DIs virus had the same temperature sensitivity as the parent virus, while two other strains had a higher ceiling temperature (Table III).

Table I. *Host range of DIs and DIE in cultured cells*

(DIE: CAM-passed original Dairen-I (DI) strain. HEK: Primary human embryo kidney cells. HEL: primary human embryo lung cells. MK: cynomolgus monkey kidney cells. RK: primary rabbit kidney cells. ESK: swine embryo kidney cells.)

Cells	DIs		DIE	
	CPE	Growth of virus	CPE	Growth of virus
HeLa	−	−	+ + +	+ + +
FL	−	−	+ + +	+ + +
HEK	+	+	+ + +	+ + +
HEL	+	± ~ −	+ + +	+ + +
MK (primary)	−	±	+ + +	+ + +
MK (continuous)	−	−	+ + +	+ + +
Chick embryo cells	+ + +	+ + +	+ + +	+ + +
RK	−	−	+ + +	+ + +
Guinea-pig kidney	±	+	+ + +	+ + +
ESK (continuous)	−	−	+ + +	+ + +
L	−	−	±	±

Table II. *Comparative titration of vaccinia virus strains and a mutant*

(DIs-25: the 25th CAM-passage of the DIs virus. DIE-24: the 24th CAM-passage of the DI virus. Lister (E-1): the 1st CAM-passage of the Lister (Elstree) virus. TCID 50: 50 % tissue culture infective dose. HAd: haemadsorption. p.f.u.: plaque-forming unit. po.f.u.: pock-forming unit. Infectivity was expressed as ^{10}log units per ml. ND: not done.)

Host	Chick embryo cells		CAM	Human embryo kidney cells		HeLa cells	
Method	TCID 50	HAd	pfu	po.f.u.	TCID 50	HAd	TCID 50	HAd
DIs-25	8·2	8·2	8·5	7·7	4·0	5·7	⩽1·7	4·2
DIE-24	8·7	8·7	8·2	8·3	8·7	8·7	ND	ND
DIs-26	—	—	8·1	ND	4·9	5·5	—	—
DIE-25	—	—	8·5	8·8	>7·7	>7·7	—	—
Ikeda (calf lymph)	—	—	7·7	7·8	6·2	8·0	—	—
Lister (E-1)	—	—	8·3	8·8	6·2	7·5	—	—

INOCULATION OF CYNOMOLGUS MONKEYS WITH THE DIS VIRUS BY VARIOUS ROUTES

Cynomolgus monkeys were inoculated with a high dose of the DIs virus by intracerebral, intravenous or cutaneous route. As shown in Table IV, two monkeys inoculated with the DIs virus intracerebrally showed a rise of body temperature from 3 to 6 days p.i., being followed by lassitude and anorexia, from which they appeared to recover later. Two other monkeys inoculated intra-

Table III. *Temperature-sensitivity of DIs plaque formation on chick embryo fibroblasts*

(Replicate CE bottle cultures were inoculated with an appropriate dilution of each virus and aliquots of infected cultures were incubated in a water bath regulated to a specified temperature. ϕ: average diameter of plaques.)

Virus strain	Temperature (°C)	Plaque formation: Plaques/bottle	Plaque formation: % of 35 °C	Descriptions
DIE	35·0	*ca.* 200	100	Typical vaccinia plaque (2·5 mm ϕ)
	38·5	99	*ca.* 50	Discrete, but smaller than at 35 °C (1·5 mm ϕ)
	40·0	0	0	No plaque-like lesion
DIs	35·0	52	100	Typical DIs plaque
	38·5	25	48	Typical DIs plaque
	40·0	0	0	No plaque-like lesion
N.Y. Board of Health	35·0	87	100	Typical vaccinia plaque
	38·5	26	30	
	40·0	15	17	
Lister	35·0	83	100	
	38·5	41	49	
	40·0	15	18	

venously remained normal. These animals were sacrificed 10 days p.i. Two other monkeys inoculated by multiple pressure showed a small area of reddening at the inoculation site from 6 to 8 days p.i. and were sacrificed 30 days p.i. Histological findings of the brain of the monkeys inoculated intracerebrally revealed leptomeningitis and ependymitis without destruction of parenchyma as confirmed by Hashizume *et al.*(1). Those inoculated intravenously showed no specific abnormality in the central nervous system (CNS). In another experiment three monkeys inoculated intracerebrally remained apparently normal, but the histological findings of the brain were not so different from those in the preceding experiment. HI antibody of the serum of the monkeys obtained at autopsy indicated that the multiplication of virus actually took place in those inoculated intracerebrally. Those inoculated intravenously or by multiple pressure showed an equivocal HI antibody response. Attempts to isolate virus from the CNS and other organs of the animals inoculated intracerebrally or intravenously were not successful.

IMMUNIZATION OF MONKEYS WITH THE DIS VIRUS AND CHALLENGE WITH SMALLPOX VACCINE

Preliminary experiments indicated that the inoculation of cynomolgus monkeys with the DIs virus at several spots elicited the production of neutralizing antibody(5). In the present experiment only a single shot was given to three groups

Table IV. *Inoculation of cynomolgus monkeys with DIs strain of vaccinia virus*

(Intrathalamic inoculation was made into the right-side portion. Attempts of virus isolation from 10 % homogenates of the brain and spinal cord of monkeys 7638, 8321, 9698 and 9707 and from 10 % homogenates of the spleen, lung, kidney and liver of monkeys 8325 and 8328 were not successful.)

Route and dose of inoculation	Monkey			Days p.i. sacrificed	Clinical signs	Serum HI	
	Code	Sex	B.W. (kg)			Antibody before inocul.	Titre at autopsy
Virus: Crude CAM homogenate (DIs-24, 25 pooled) $10^{8\cdot0}$ TCID 50/ml							
Intrathalamic 0·4 ml	7638	F	2·80	10	Fever (3–6 days p.i.) slight anorexia (3–7 days p.i.)	4	80
Intracisterna 0·25 ml	8321	F	2·71	10	Fever (3–5 days p.i.), lassitude and anorexia (6–10 days p.i.)	<4	80
Intravenous 1·0 ml	8325	M	2·30	10	None	<4	<10
	8328	M	2·20	10	None	<4	10
Cutaneous (multiple pressure)	8322	M	2·54	30	Local reddening (6–8 days p.i.)	<4	10
	8323	F	2·54	30	Local reddening (6–8 days p.i.)	<4	<10
Virus: fluorocarbon-treated (2 ×) CAM homogenate (DIs-26) $10^{7\cdot8}$ pfu/ml							
Intrathalamic 0·5 ml	9698	F	2·80	10	None	<2	16
	9699	F	3·24	10	None	<2	32
Intracisterna 0·25 ml	9707	F	2·08	10	None	<2	16

Virus isolation from 10 % homogenates of the brain and spinal cord of monkeys 7638, 8321, 9698 and 9707 and from 10 % homogenates of the spleen, lung, kidney and liver of monkeys 8325 and 8328 was negative.

Table V. *Local reaction of the monkeys after cutaneous inoculation with smallpox vaccine*

(Cynomolgus monkeys immunized with a single dose of the DIs virus as specified in the Table were inoculated 13 weeks later with the regular calf-lymph vaccine of Ikeda strain (2×10^8 po.f.u./ml) by multiple pressure (4 pressures with a bifurcated needle). Three untreated monkeys kept in the same room during the immunization period and 2 fresh monkeys served as non-immune controls.)

Group	Pretreatment with DIs virus	Identity of monkey	Diameters of induration or swelling (mm) at days after smallpox vaccine		
			4	7	9
I	Subcutaneous	9423	7·5	13·0 (A)	9·0 (A)
	1·0 ml	9438	3·0	3·5 (—)	2·0* (—)
	($10^{7.7}$ pfu)				
II	Intradermal	9429	6·0	12·0 (—)	10·5† (—)
	0·2 ml	9430	4·0	11·0 (B)	7·0† (B)
	($10^{7.0}$ pfu)	9431	6·0	16·5 (—)	11·0† (—)
III	Intramuscular	9425	6·5	12·0 (A)	8·0† (B)
	1·0 ml	9427	6·0	10·0 (—)	7·0† (—)
	($10^{7.7}$ pfu)	9428	6·0	10·5 (B)	7·5† (—)
IV	None (room mate)	9433	5·0	8·5 (A)	11·0 (A)
		9435	5·0	12·5 (—)	19·0 (—)
		9436	6·0	13·5 (B)	11·0 (B)
V	Non (fresh)	8887	5·5	12·0 (A)	11·0 (A)
		8988	7·0	15·0 (B)	10·5 (A)

(A) Swelling of an axillary lymph node of a sparrow- to quail-egg size.
(B) Swelling of an axillary lymph node of a bean to soybean size.
(—) No swelling of axillary lymph node.
* Skin lesion almost completely cured.
† Slight flat swelling without induration.

of monkeys as shown in Table V. Thirteen weeks later they were inoculated with a commercial lot of calf-lymph vaccine by multiple pressure. Local reaction of the monkeys pretreated with the DIs virus was not so different from that observed in non-immune animals. However, those immunized intradermally or intramuscularly showed a slightly accelerated local reaction.

ANTIBODY RESPONSE OF DIS-IMMUNIZED MONKEYS

In DIs-immunized monkeys the HI antibody appeared 2 weeks p.i., and reached its height about 3 weeks p.i. and then began to decline. By the inoculation with smallpox vaccine, however, the immunized animals responded with an earlier rise of HI antibody. Serum neutralizing antibody was not detectable in DIs-immunized animals up to the time of challenge with smallpox vaccine. After challenge, however, the appearance of circulating neutralizing antibody was also

Table VI. *Neutralizing antibody response of normal or DIs-immunized monkeys to cutaneous inoculation of smallpox vaccine (Ikeda strain)*

(Monkeys were bled at specified time intervals and the serum obtained was inactivated at 56 °C 30 min before use. Neutralizing antibody was measured by plaque reduction of DIE virus in CE monolayers under agar overlay and the titre was expressed as reciprocal of the serum dilution which reduced the number of plaques to 50 % of those produced by the control virus.)

Monkey group and immunization	Monkey code	Neutralizing antibody titre of serum at weeks p.i.		
		1	2	4–5
(I) Subcutaneous	9423	63	550	<25
	9438	<25	173	33
(II) Intradermal	9429	<25	125	119
	9430	<25	376	45
	9431	28	146	<25
(III) Intramuscular	9425	42	745	252
	9427	72	315	93
	9428	<25	30	<25
(IV) None (room mate)	9433	<25	<25	<10
	9435	<25	29	11
	9436	<25	<25	13
(V) None (fresh)	8887	<25	<25	40
	8987	<25	<25	25

much accelerated in immunized animals and their titres were considerably high. A high level of neutralizing antibody, however, did not seem to continue long. On the contrary, the appearance of serum neutralizing antibody in non-immune animals was delayed and their titres were low (Table VI).

PREPARATION OF A STOCK DIS VIRUS FREE FROM AVIAN LEUCOSIS CONTAMINATION

Since the DIs virus was passed exclusively through conventional eggs, it was considered necessary to prepare a stock virus free from avian leucosis contamination. Because vaccinia virus is resistant to ethyl-ether and sensitive to avian leucosis virus, DIs-infected CAM homogenate was treated twice with fluorocarbon, followed by ethyl-ether treatment. The virus preparation was then passed through avian leucosis-free eggs three times, at each passage the inoculum being treated with fluorocarbon and ethyl-ether. The 3rd passage virus was assayed by Rubin test and proved as avian-leucosis-free. Preliminary experiments on the inoculation of the DIs stock virus thus prepared to non-immune children by multiple pressure or intradermal inoculation indicated that the local reaction was minimal with mild erythema with or without induration. Vesicle formation

was rarely observed and no systemic reaction was noticed. Further studies are now under way to find a suitable method to give non-immune children the basic immunity with this virus.

DISCUSSION

A number of vaccinia virus mutants with modified pathogenicity in experimental animals have been reported, but only a few were examined for availability in man as a possible candidate of attenuated smallpox vaccine. Rivers and his associates(**8–11**) passed the New York City Board of Health strain of vaccinia virus through chick embryo tissues and noticed the attenuation of pathogenicity both in rabbit and man. Kempe *et al.*(**4**) and Tint & Bierly(**14**) used the 1st revived strain of Rivers (CVI) and Noordaa *et al.*(**7**) used the second revived strain for the primary vaccination of children or military recruits. Their clinical observations as well as biological properties of the CVI virus(**1–3, 13**) may suggest that these viruses are attenuated to a certain degree but not to a great extent. Further attenuated strains of vaccinia virus were reported by Stickl & Hochstein-Mintzel(**12**) and the present authors. The MVA virus (or FHE virus(**6**)) is markedly attenuated in pathogenicity in rabbits and almost completely avirulent by direct inoculation into CNS of laboratory animals(**12**). These characteristics appear quite similar to those of the DIs virus, although they differ from each other in some other properties. Very weak antibody responses in monkey and man inoculated with either virus may suggest a quite limited availability of these viruses as smallpox vaccine, but experimental data appear to indicate that the basic immunity may be induced by these viruses, which may render the reaction by the regular vaccine more or less milder.

SUMMARY

A mutant (DIs) of the Dairen-I strain of vaccinia virus was obtained, which is a host cell-dependent conditional lethal mutant. It was found that the mutant can grow, though much less luxuriantly than the parent virus, in primary human embryo kidney cells. Inoculation of cynomolgus monkeys with the DIs virus by various routes indicated that the mutant was highly attenuated but induced weak immunity to cutaneous inoculation of vaccinia virus. A stock virus preparation was prepared with DIs-infected CAM homogenate, which proved free from avian leucosis contamination. A possible availability of the mutant as an immunogen to induce the basic immunity to smallpox vaccination was discussed.

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Discussion

MARENNIKOVA (USSR) I would like to know what kind of pocks you chose for isolating your mutant. Was it a white pock?

TAGAYA (Japan) The original virus preparation passed through 1-day eggs was not cloned.

RONDLE (UK) I have listened to the last five papers with interest but I am slightly confused. We know that vaccinia strains differ in biological properties. However, do the different strains represent different mutants or only attenuated strains? If only attenuated strains, then 'vaccinia' has one common virus genome which is expressed in different ways depending on conditions of growth. Hence growth conditions for vaccine production must be very carefully controlled. If the different strains are mutants then the implications are wider.

Either the vaccinia strains are mixed populations where selection occurs over multiple

egg- or tissue-culture passages, or there is a rapid dissociation of vaccinia strains leading to mixed populations of virus genomes. In the first case a series of single pock isolates should lead rapidly to the establishment of stable virus strains. This would be highly desirable for vaccine production. If the second case were true it would be imperative to prepare vaccine from virus pools not more than two or three passages away from the seed virus, otherwise reproducible materials could not be produced. It is perhaps significant that monkeypox produces variants with a frequency of approximately $2-5/10^5$ pocks. 'Field' cowpox produces variants with a higher frequency. Nevertheless it is tempting to think that most 'vaccinia' strains are mixtures of virus genomes. In a few cases – possibly 'Elstree' for example – one genome has been selected out to give a stable strain of highly reproducible behaviour.

TAGAYA (Japan) The attenuated pathogenicity of a certain vaccinia strain to experimental animals is also a phenotypic expression of the viral genome and consequently is considered as a mutation from the original virus. Since vaccinia virus is considered as laboratory virus, we have no prototype vaccinia virus, although the Elstree strain was established as the reference virus by the WHO. As Joklik stated, I think it is reasonable that every vaccinia virus strain should be characterized by its strain name, because every strain differs from others in some points.

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INACTIVATION AND POTENCY TESTS WITH VACCINIA VIRUS

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Vaccinia antigen, an inactivated vaccinia virus preparation, has been in use in Germany for more than 10 years. The product is intended for the prevention of untoward side-effects after live smallpox vaccination (Herrlich).

National requirements for safety and potency are similar to the ones for inactivated polio vaccines. The problem of inactivation of the DNA virus and the measurement of potency have never been discussed in public. The following contribution tries to furnish whatever information was obtained on these subjects during manufacture of more than 600000 doses. Inactivation of the virus grown on rabbit tissue culture was performed with formaldehyde (HCHO). The loss of infectivity is temperature-dependent as shown in Fig. 1. Infectivity decreased at 37 °C about 4 times faster than at 20 °C. At both temperatures the tailing effect is quite pronounced for both curves, i.e. inactivation of the virus at a surplus of HCHO molecules does not follow a so-called first-order reaction. More than one hit of HCHO is necessary to inactivate the virus. To those familiar with inactivation of other viruses this is not at all surprising, since the same situation exists for example for polio virus. Fig. 2 compares the data for both viruses. It is quite obvious that both processes follow identical biochemical rules as far as loss of infectivity is concerned. Our national requirement for proof of inactivation is therefore the same as for a monovalent poliovirus pool: 500 ml of the inactivated virus suspension should not contain any tissue-culture infective virus. The significance of this requirement is not based on sufficient evidence on the relation between tissue culture or animal infectivity and the infectivity for man. It has been a long argument whether inactivated vaccinia virus can or cannot produce haemagglutination-inhibiting antibodies (HAI) in animals. As for rabies virus, some residual infectivity was regarded as prerequisite for the induction of antibodies. When we studied this question we found a dose–response relation as demonstrated in Fig. 3. It can be seen that with increasing virus concentration the increase in antibody titres follows as a linear function. No surviving virus infectivity is needed; the inactivated antigen adsorbed to a constant concentration of $Al(OH)_3$ is quite potent in inducing HAI antibodies in guinea-pigs.

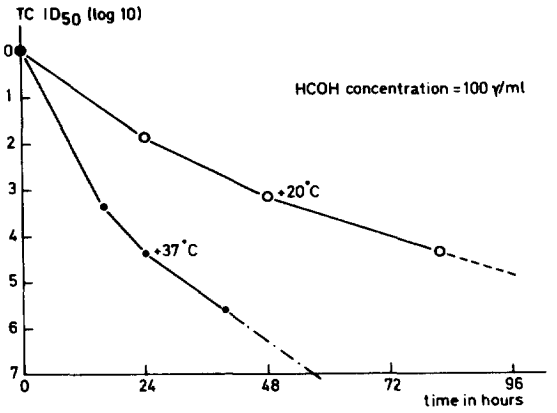

Fig. 1. Inactivation of vaccinia virus. Loss of infectivity at two different inactivation temperatures.

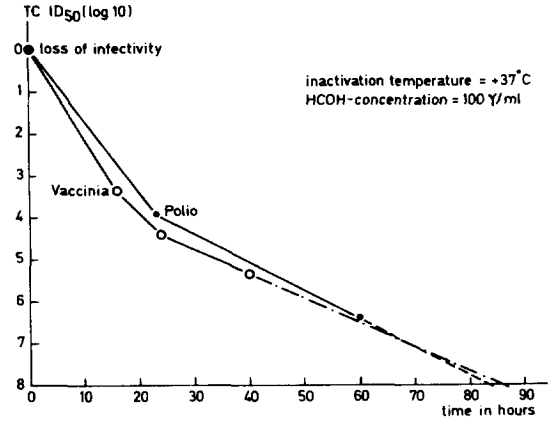

Fig. 2. Inactivation of vaccinia virus compared with polio virus type 1.

Discussion

HOCHSTEIN-MINTZEL (West Germany) What was the initial titre of the suspension before inactivation and how much vaccine had to be applied and how often to cause a serological response?

HENNESSEN (West Germany) The initial titre was 10^7 before inactivation and the animals received one dose of 0·5 ml. They were bled after 3 weeks.

APPLEYARD (UK) It is generally accepted that haemagglutinin is not a component of the virus particle but is separate from and smaller than the virus particles. Is it not possible that the HI-antibody is found in response to free haemagglutinin rather than to the inactivated virus? If the virus had been rigorously purified, do you believe that an HI antibody response would still have occurred?

HENNESSEN (West Germany) The virus has not been purified to obtain pure virions and no subunits. So it cannot be excluded that such subunits as free haemagglutinin are present in the product.

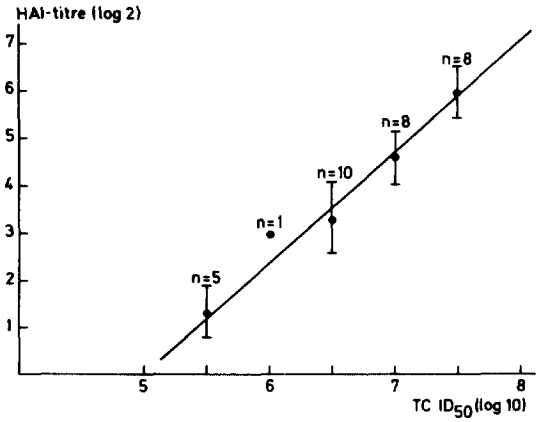

Fig. 3. Vaccinia antigen. Potency in guinea-pigs vs. virus-titre before inactivation.

RONDLE (UK) There are two reports in the literature that haemagglutinin forms part of the virus particle. This work has not been confirmed, but the possibility must be borne in mind when considering Dr Appleyard's comments.

HENNESSEN (West Germany) I may add here that if you disrupt the virus particle by some chemical means, the ability to induce haemagglutination-inhibition antibody disappears, but here again this may be due to the action on the subunits which are present in the first place.

NETTER (France) What is the keeping quality of such vaccine? Is it the same as for other vaccines? Does it still stimulate antibody after 2 years storage at 4 °C?

HENNESSEN (West Germany) It does not lose its antibody inducing activity for 2 years at refrigerator temperature.

EHRENGUT (West Germany) Did you observe that the antigenicity of your preparation inactivated at 37 °C was lower than that of a preparation inactivated at lower temperatures?

HENNESSEN (West Germany) Yes, we observed this, as was to be expected from observations made by Dr Sven Gard's Swedish poliovirus vaccine production.

STICKL (West Germany) Proof of the antigenicity is also possible using the Henkin test, a skin test with trypane blue dye intravenously. In this way it might be possible to avoid testing only the haemagglutinin without the virus antigen. In the skin tests a tissue culture virus without antihaemagglutinin-inducing qualities has been used by Stickl, Vanek and Münz.

HENNESSEN (West Germany) No comment.

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MOUSE PATHOGENICITY OF SOME VACCINIA VIRUS STRAINS

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In 1968 the Lister strain of smallpox vaccine was introduced in Hungary for primary vaccinations instead of the formerly used Budapest strain, derived from Berlin in 1927. On the basis of very favourable experience of a 3-year period, all vaccinations have been carried out with the Lister strain since the beginning of 1972. There are many papers dealing with the reactogenicity and immunogenicity of the different vaccine strains. In Hungary Erdős(**3**) and Nyerges(**9**) have analysed the results obtained in human vaccinations and in immunizations of laboratory animals. They found less reactogenicity in man and a higher immunogenicity in humans and animals vaccinated with the Lister strain, as compared to the Budapest strain vaccine. Despite these findings it seemed necessary, from the standpoint of the vaccine producer, to control his strain in its consecutive passages, especially its reactogenicity. In our experiments we applied the intranasal infection of suckling mice published by Marennikova(**8**) and Krag Andersen(**7**). The pathogenicity of Budapest, Lister and EM63 strains was compared. The latter known by its very low pathogenicity in suckling mice served as control.

MATERIALS AND METHODS

Each strain used was a freeze-dried calf dermovaccine, prepared by ourselves, partially purified by Freon 113 treatment and differential centrifugation. The Lister vaccine-strain was obtained from the Lister Institute of Preventive Medicine, London, and has been maintained in alternative passages in rabbits and calves. Up to now it has undergone three such passages. The EM 63 strain was sent by Dr Marennikova, Moscow; it was passed twice in rabbits and twice in calves. The Budapest strain had undergone many passages in rabbits and calves. Suckling mice from the CFLP strain were inoculated intranasally with 0·01 ml of the vaccine suspensions in saline. At the same time at least four litters were given the same inoculum. The animals became ill on the 4th–5th day of infection. The mortality was recorded daily until the 14th–21st day.

RESULTS

(1) *The role of the age of animals in mortality* The mice were infected with the Lister strain ($10^{5·3}$ pfu per mouse) on different days after birth and observed during a 21-day period. The rate of the mortality was expressed as a percentage. The sensitivity of the animals gradually decreased from the 2nd day of age until

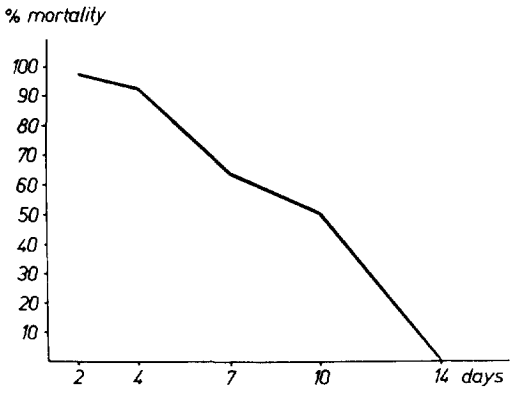

Fig. 1. Mortality of suckling mice inoculated in different ages of life. Inf. dose: $10^{5\cdot3}$ pfu/mouse.

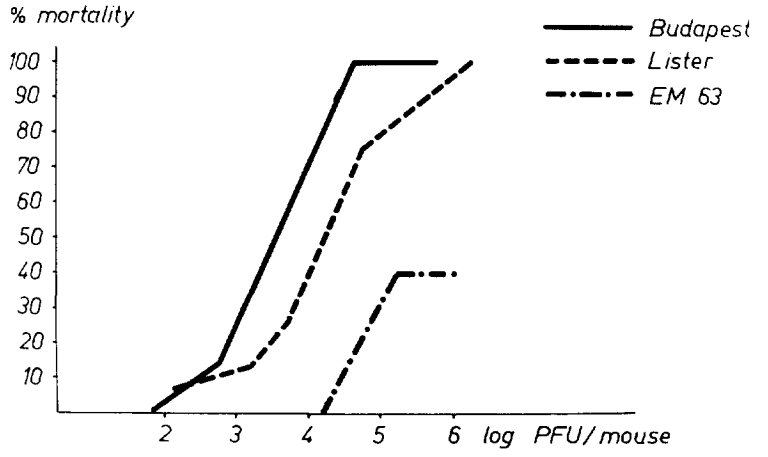

Fig. 2. LD values of different vaccinia virus strains.

the 14th day. It was concluded that the optimal age to infect was the 2nd day after delivery.

(2) *Infectivity titration in mice* The mortality caused by the different, logarithmic virus dilutions was recorded as a percentage.

(*a*) Budapest strain: the LD 50 was $10^{3\cdot5}$ pfu/mouse.

(*b*) Lister strain: the LD 50 was found at $10^{4\cdot2}$ pfu/mouse.

(*c*) EM 63 strain: at $10^{5\cdot3}$ pfu/mouse the mortality reached its maximum (40%), so the LD 50 value could not be determined.

(3) *Time of death caused by different strains* Two-day-old mice were infected with the same virus doses of the three strains ($10^{5\cdot3}$ pfu per mouse). The mortality was recorded daily as the percentage of the infected mice. The highest mortality

Table I. *Virus titres in the organs on different days after infection*

	Day	Virus strain		
		Budapest	Lister	EM 63
Lung	2	7·0	6·6	7·1
	5	8·4	8·4	7·9
	7	8·4	8·5	7·4
	9	+	8·6	7·6
Blood	2	2·9	2·4	2·1
	5	4·7	3·0	1·7
	7	4·9	5·2	3·2
	9	+	2·8	< 1·0
Brain	2	3·0	2·0	2·0
	5	5·8	4·4	2·5
	7	6·6	6·0	3·5
	9	+	4·9	2·8
Spleen	2	3·8	3·0	2·9
	5	6·9	5·4	2·2
	7	6·8	3·8	< 1·0
	9	+	4·0	< 1·0
Liver	2	3·0	2·0	2·6
	5	6·3	3·9	2·6
	7	4·5	3·7	2·0
	9	+	2·2	< 1·0
Kidney	2	4·0	3·7	2·5
	5	6·8	6·8	6·4
	7	6·2	6·8	6·0
	9	+	6·4	3·7

Infecting doses: $10^{5\cdot3}$ pfu mouse.
Virus titres: ^{10}log pfu/g.
+ : died.

was reached on the 8th day with the Budapest strain and on the 12th day with the Lister strain. The mortality using the EM 63 strain reached its maximum (40 %) on the 8th day.

(4) *Virus titres in the organs in the course of the infection* Two-day-old mice were infected with a constant dose of each virus strain ($10^{5\cdot3}$ pfu per mouse) and samples of five mice were sacrificed on the 2nd, 5th, 7th and 9th days of infection. The organs were homogenized and the virus titres were determined on CAM. The virus titres of the lung, blood, brain, spleen, liver and kidney were titrated and calculated to 1 g of wet weight (Table I).

In the lung, high titres were observed with each virus strain, although the EM 63 produced somewhat lower titre than the other two. In the blood there were significant differences on the 5th day and the same results were obtained

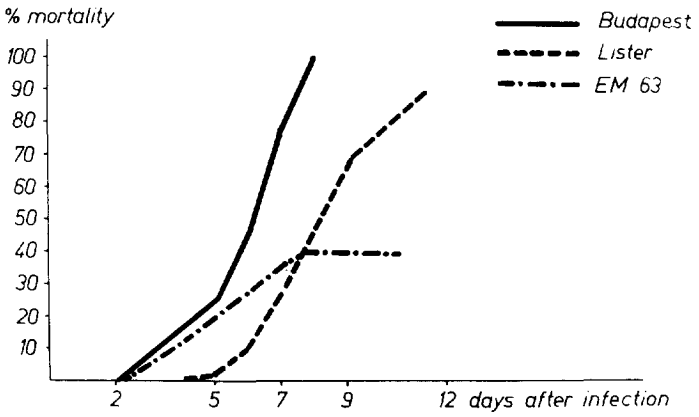

Fig. 3. Mortality rates on different days after infection. Inf. dose: $10^{5\cdot3}$ pfu/mouse.

in the spleen and liver. The Budapest strain produced the highest, the EM 63 the lowest titres; the Lister strain occupied an intermediate position. It is noteworthy that in these latter two organs the EM 63 vaccine virus was not demonstrable from the 7th and the 9th day respectively. No differences between the strains were observed in the kidney, where each strain produced high titres.

In order to elucidate the pathogenesis, we tried to demonstrate the virus antigen in the organs by the fluorescent antibody technique using a hyperimmune bovine gamma globulin conjugate, but we found positive fluorescence in the lungs only. The results were similar with each strain: fluorescence was demonstrable in the small bronchi. In other organs including the kidneys we could not observe any specific fluorescence. This finding does not exclude the virus multiplication in these organs taking into account the great differences in their virus titres compared with the lung.

(5) *Infection of macrophages* Macrophages are known to play a primary role in taking up and disseminating viruses. We tried to infect mouse macrophages *in vitro* with our virus strains. Adult mice of 14–16 g were stimulated with intraperitoneal injection of 10% pepton solution and peritoneal macrophages were cultured in bottles of 20 mm diameter in Hanks solution containing 10% calf serum; 10000 cells were attached to the glass per bottle and they were infected with $10^{5\cdot0}$ pfu of virus. After 2 h adsorption the unadsorbed virus was removed by washing the cells three times with medium and once with 1% solution of hyperimmune bovine gamma globulin. The infected macrophages were then overlaid with 200000 cells of a continuous monkey kidney cell line(**11**). The plaques originating from the infected cells were counted 48 h later.

The number of infected macrophage cells was found to be 1 per 500 for each virus strain.

DISCUSSION

In the present experiments the suckling mouse pathogenicity test has shown a parallelism with the vaccination reactions in humans, regarding the Budapest and Lister strains. The results are consistent with the less-intensive reactivity of the Lister strain(**3**). The test seems to be able to control vaccines under preparation. We have no experience with other strains, which may give divergent results, and we are aware of the fact that the results are not applicable directly to man, and the exact evaluation of the strain-pathogenicity must be based on several, different methods. However, our experiments seem to verify the lower pathogenicity of the Lister strain.

Our efforts in studying the pathogenesis in animals and the divergent pathogenicity of the three strains remained rather unsuccessful. There is an active virus multiplication in the lung followed by viraemia and the virus is present in the organs, but to a different degree, when using different strains. These differences seem to be parallel with strains of different pathogenicity. It seems very probable that the more invasive character of one of the strains is connected with its higher affinity to certain cell types. Evidence has been presented that macrophages obtained from mice of genetically different strains were differently sensitive to the same virus(**6**) and on the other hand attenuated viruses were less infective to macrophages than wild ones(**1, 2, 10**), although both could be harboured by macrophage cells for a longer time. In our experiments adult mouse macrophages could be infected *in vitro* with each strain with equal ease. This confirms that they may play a role in the virus infection, but does not elucidate the causes of the different invasiveness, although there remains the possibility that suckling mouse macrophages could be different in their sensitivity from the adults, as found by others(**4, 5**). There may be other factors in the different pathogenicity of the strains, and to investigate them may be helpful to a better understanding of the very unclear mechanisms of vaccination reactions and complications.

SUMMARY

After intranasal infection of suckling mice the highest mortality was caused by the Budapest, lower by the Lister and very low by the EM 63 vaccine.

Significant differences of the same order were demonstrated in the virus titres in the blood, brain, spleen and liver of the animals. Thus the lower pathogenicity of the Lister strain against the Budapest strain, observed by others as lower reactogenicity in clinical surveys, has been confirmed by this test.

With the exception of the lungs, no virus could be demonstrated in any organs of the infected animals by the fluorescent antibody technique.

Adult mouse macrophages *in vitro* could be infected with each strain with equal ease.

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Discussion

MARENNIKOVA (USSR) I would like to emphasize that your data show close correlation with the data obtained by us in respect to the behaviour of the different strains. I am glad you investigated this subject from the other side.

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NON-INFECTIOUS SMALLPOX VACCINE IN THE PROPHYLAXIS OF POSTVACCINIAL ENCEPHALITIS

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The aim to prevent postvaccinial encephalitis completely has by no means been achieved. Many trials, including attenuated vaccines, change of vaccinia strains, dilution of vaccine or hyperimmune serum-prophylaxis and preimmunization with non-infectious vaccine, have been done. Although the last-mentioned two procedures have definitely reduced the incidence of this severe complication of Jennerian vaccination, there is no possibility of preventing encephalitis with absolute security.

Because the pathomechanism of postinfectious encephalitis is unknown, the rationale for a way of prevention is unsatisfactory. Hyperimmune serum is said to shorten the viraemic stage after vaccination, which may be connected in some way with the onset of encephalitis.

Postinfectious encephalitis occurs very seldom in revaccinees and the conclusion has been drawn that some residual immunity may be of importance to prevent the development of encephalitis. Since encephalopathies arise only after the use of live virus, the idea of preimmunization with a non-infectious vaccine to induce some kind of immunity was the consequence. (Verlinde, Herrlich, Ehrengut, Beunders (**23**, **12**, **6**, **3**)).

In fact, in subsequent papers it could be demonstrated(**6**, **7**, **9**, **10**) that killed vaccine acts like an antigen. Clinically it induces a marked acceleration of the pustule in the preimmunized primary vaccinee, comparable with the faster healing of the local lesion of the revaccinee. In addition, vaccinial allergy has been shown to develop already 2 days earlier in the preimmunized vaccinee(**10**). At the same time vaccinial antibodies can be found earlier after the combined method. The antigenicity of the non-infectious vaccine can be demonstrated also in the revaccinee, who shows a rise in HI-antibodies after stimulation(**4**, **6**, **8**, **14**, **21**).

Lindenmann & Buser(**17**), Dostal(**5**) in addition to Kaplan(**14**), postulated a field trial with non-infectious vaccine. In our country, such a trial cannot be done in view of ethical reasons, since different European studies(**2**, **13**, **22**) have revealed that the older primary vaccinee is more prone to succumb to postvaccinial encephalitis. Doubts about a higher frequency of postvaccinial encephalitis in the older subject have arisen from American sources. Lane & Millar(**16**) pointed out that in the US Army 2 million soldiers have been vaccinated against smallpox without any fatal outcome. For reassurance, we asked Dr Alexander (Director Institution of Preventive Medicine, US Navy), who told

Table I. *Preimmunization with non-infectious smallpox vaccine and age of the vaccinees*

	Age (years)				
	0–3	3–6	6–9	9–12	Over 12
No. of vaccinees	7779	6064	3079	3984	10057

us that in fact the mandatory vaccinations in the US army and Navy had not given any concern, but he admitted that the majority of the soldiers must have been revaccinees(1). In addition, he suggested that differences in smallpox vaccine strains may also be of some importance. The study of Neff(19) has shown that almost all Americans have been immunized against smallpox during their life.

One cannot compare, as Kaplan(15) did, the incidence of postvaccinial encephalitis in different countries with the aim of evaluating prophylactic measures against postvaccinial encephalitis. One always has to consider carefully the bias of all statistics. In a country with mandatory notifications of vaccinial injuries and a system for recompensation by the state as in the Federal Republic of Germany, the public vaccinator gets a pretty good idea of what is going on in his district.

Since 1957 we have been working with non-infectious vaccine in order to prevent postvaccinial neural complications. Therefore we feel it would be worth while to report our experience with this method.

In Bremen and Hamburg preimmunization with non-infectious smallpox vaccine has been done in the numbers of primary vaccinees shown in Table I. Among the 23148 subjects over 3 years old, there was 1 case of encephalitis. In comparison, a follow-up on 488 cases of postvaccinial encephalopathy(11) and Federal Republic of Germany, period 1956–65), showed 1 case of encephalopathy among 25000 children under 3 years of age *without* preimmunization. Thus, by preimmunization with non-infectious vaccine we seem to reach the frequency of encephalopathy in the young child. Since the primary vaccination (without preimmunization) of children over 3 years old has been cancelled by legislation in our country, we are only able to add 60 children of 12 years of age as a control group, vaccinated primarily in Hamburg due to misinterpretation of scars. This group included already one serious case of encephalomyelitis. These figures are in harmony with those of Berger & Puntigam(2) (1 encephalitis among 105 vaccinees). In a follow-up study of cases of postvaccinial encephalitis in Bavaria(13: period 1952–4), amongst 5727 children between 4 and 12 years of age, 6 cases (1 in 954 primary vaccinees) were observed.

There is still another possibility of studying the value of preimmunization with non-infectious vaccine. In the Federal Republic all notifications of vaccinia injuries are reported to the 'Bundesgesundheitsamt'. This office gave us the opportunity to study all records concerning injuries in subjects preimmunized with non-infectious vaccine. Thus we were able to collect eight cases of postvaccinial encephalitis occurring in persons over 3 years old. There was no fatal

case, only one patient showed sequels. In order to find out the amount of vaccinees vaccinated on a voluntary basis, we asked the two producers of non-infectious vaccine in Germany for the amount of doses of non-infectious vaccine sold during that period. They distributed 550000 doses of 'Vaccinia-Antigen'. This vaccine is also used in older subjects to boost their antibodies in order to mitigate the local reaction following revaccination.

If we assume that only half the amount of the vaccine has been used for primary vaccinees over 3 years of age (an amount that still seems very small), there was 1 case of encephalitis in at least 28125 subjects. This figure is similar to that of an investigation of Rohde(**20**) in East Germany. He found 1 case of encephalitis among 20000 subjects over 3 years old, preimmunized with non-infectious vaccine.

This result is fairly comparable with that of Nanning(**18**) in the Dutch Army. Nanning reported 3 encephalitides among 53630 recruits who were primarily vaccinated under the cover of vaccinia-hyperimmunglobulin, i.e. 1 case of encephalitis per 17876. On the other hand, he reported 1 encephalitis per 4000 untreated controls.

We can therefore conclude that the simultaneous application of antivaccinia-hyperimmunglobulin as well as the pretreatment with non-infectious vaccine before primary vaccination of older individuals is a suitable prophylaxis of postvaccinial encephalitis.

SUMMARY

Since 1960 non-infectious smallpox vaccine was used for the prophylaxis of postvaccinial encephalitis in 23148 primary vaccinees over 3 years of age in Bremen and Hamburg. One case of encephalitis without sequels occurred (0·43 per 10000). Furthermore, neural complications of vaccinations in the Federal Republic of Germany have been investigated. In about 10 years 550000 doses of killed vaccine have been sold; eight cases of postvaccinial encephalitis were registered in this period. Assuming that only half the sold vaccine had been used for older individuals the frequency of postvaccinial encephalitis was calculated as 1 in 28125 primary vaccinees over 3 years of age (0·36/10000).

We are grateful to Prof. Dr H.-J. Weise, Bundesgesundheitsamt Berlin in providing us with the notifications of vaccinial injuries in the Federal Republic of Germany.

We thank also Dr P. Geissler, Chief Health Officer, Gesundheitsamt Bremen, for the vaccination statistics of Bremen.

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Discussion

MARENNIKOVA (USSR) What interval between preimmunization and inoculation of live vaccine do you now use in Germany?

EHRENGUT (West Germany) We usually have a short interval of 8 days but we do not mind if it is longer and the interval is 6 weeks. There is no difference in the occurrence of vaccinia ulcers with short or long intervals. In tiny babies we never take any other prophylactic measures. Only if the parents are very anxious we sometimes give gamma-globulin.

EDSALL (UK) I find it difficult to fully accept interpretations of data which compare results in circumstances that are not strictly and clearly comparable and I think I can make my point clear by saying that I have never yet understood how to explain the difference between the military recruit rate in the Netherlands of 1 in 4000 and the very clearly established rate, if you can call it a rate, in US military recruits over the past 15 or 20 years of 1 in several hundred thousands unless you assume that the major difference is perhaps in the vaccine strain as you see in the recent data from the Netherlands. Therefore, unless you have a clear situation where the before group and the after group are identical or two simultaneous groups are vaccinated, it is very difficult to know how one can interpret this sort of data.

EHRENGUT (West Germany) The incidence found depends a great deal on how hard you look. I know of a paper of Agnew, who found among 4500 primary vaccinees in the British Royal Navy one case of encephalitis, and this is very similar to the data of the Dutch Army.

LANE (USA) I just want to make a clarification and ask one question. As to the clarification, Dr Ehrengut mentioned about 2 million US Army vaccinees. However, of the approximately 2 million vaccinations since the Second World War, 5–10% only were primaries. No known death from encephalitis were observed in the over 2 million adult vaccinations.

As to the question: how many children fail to come back for revaccination after the killed vaccine? All of the preimmunization techniques, either killed vaccine or attenuated vaccine, may fail if a high proportion of children do not return for the second step of immunization.

EHRENGUT (West Germany) I guess that about 5% of the preimmunized subjects do not return immediately for live vaccination, but they will be vaccinated later. In infants we could see even after an interval of 1 year a local take of the accelerated type; in other words, the effect of the preimmunization can still be seen.

NEFF (USA) It will be difficult to compare the incidence of postvaccinal encephalitis from one country to another until there is consistent definition of what comprises a case of postvaccinal encephalitis.

EHRENGUT (West Germany) In our group we have given a definition of postvaccinal encephalitis. We think it is important to include also the dubious cases. At least one third of the cases in our files are dubious ones because we are not sure vaccination has been eliminated as the cause.

BENENSON (USA) Would you tell us a little more about the frequency and severity of the vaccinal ulcers you mentioned.

EHRENGUT (West Germany) The frequency of ulcer formation in preimmunized subjects is dependent on the age of the vaccinees (Ehrengut & Rechnitz: *Paed. Prax.*, in the Press): among 16466 subjects we found 149 vaccinal ulcers: 0–3 years, 0·19/100 primary vaccinees; 3–6 years, 0·25/100; 6–9 years, 0·45–100; 9–12 years, 1·94/100; 12 and more years, 1·72/100. The difference in frequency between the 6- to 9-year-old children and those 9–12 years old was highly significant (χ^2 test: $P < 0{\cdot}1\%$). This fact is explained

by the increase of 'unspecific resistance' (see Pfaundler: *Mschr. Kinderheilk.* **24**, 623 (1923)) at the age of 9–12 years. The vaccinial ulcers can be treated by desensitization with non-infectious smallpox vaccine, which induces a faster healing of the process (Ehrengut: *Pressemed.* **72**, 1957 (1964)). One important difference between the immunity elicited by attenuated and non-infectious vaccine is the fact that the vaccinee pretreated with the latter vaccine has according to my experience always a markedly accelerated reaction to the revaccination after 10 years. We explain this, based on the concept of Salk: the stronger the antigenic stimulus at the onset of an immunization, the better the persisting immunity.

STICKL (West Germany) The frequency of undue reactions such as ulcer, exanthema and strong local reaction after vaccinia antigen and virulent vaccination can be reduced by shortening the interval between giving the vaccinia antigen and vaccination with potent vaccine to 4 days instead of 8–14 days. Normally, undue local reactions after pretreatment with inactivated vaccinia virus (vaccinia antigen) occurred in 5–8 %; after we shortened the interval we hardly saw any cases.

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INTRACEREBRAL INOCULATION OF MONKEYS WITH SEVERAL VACCINIA STRAINS: AN APPROACH TO THE COMPARISON OF DIFFERENT STRAINS

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WHO requirements for Smallpox Vaccine recommend that the strain used for vaccine production should be one that has never shown a greater tendency to produce generalized lesions or lesions of the nervous system in either man or animals than other strains of vaccinia virus which have been in general use for many years, and that strains of so-called 'neuro-vaccinia' should be excluded (**8**).

Marennikova *et al.* (**6**) reported classification of the vaccinia strains into 3 grades with respect to pathogenicity from the results of intracerebral inoculation into rabbits or mice and intravenous inoculation into irradiated mice or rats, and intradermal and intratesticular inoculation into rabbits, etc. Rozina *et al.* (**10**) reported that there are pathogenic and apathogenic strains; the latter was shown to cause only mild arachinoiditis by intracerebral inoculation into monkeys.

The intracerebral inoculation test in monkeys has been adopted in requirements of live polio, measles, and rubella vaccines. The test has never been applied to smallpox vaccine. It would be necessary to carry out research to establish attenuated strains for producing smallpox vaccine.

This report will present the results of such attempts with several vaccinia strains.

MATERIALS AND METHODS

Lister, EM 63, New York City Board of Health (NYBH), Ikeda, CV 1, DIs(**11**), and Lc16 strains were used. The Lc16 strain was a progeny of cloned Lister strain passaged in rabbit kidney cells at 30 °C. The characteristics of the strain were as follows:

(1) The reproductive capacity at a high temperature (40·5 °C) was lower than that of the parent strain.

(2) Neurovirulence in rabbits was lower than that of the parent strain.

(3) Local reaction and HI antibody response by intradermal inoculation of the Lc16 strain in rabbits was almost the same as those of the parent strain.

The Lc16 strain was cultured in rabbit kidney cells at 30 °C and egg-grown virus of the other six strains were used. The virus used for inoculation was purified by the fluorocarbon treatment and sucrose density-gradient centrifugation. Portions of 0·5 ml of each of serial 10-fold dilutions of the virus were injected into one side of the thalamus of three monkeys.

The monkeys showing clinical symptoms were autopsied as soon as possible after

Table I. *Mortality of the monkeys inoculated in the thalamus with different inocula of the vaccinia strains*

Virus strain inoculated	Inoculum	Average day of death	Rate of death	LD50
	pfu			
CV 1	2×10^8	4·0	3/3	$\leqslant 6{\cdot}3 \times 10^5$ pfu
	2×10^7	5·0	3/3	
	2×10^6	7·3	3/3	
Ikeda	2×10^8	4·0	3/3	$\leqslant 6{\cdot}3 \times 10^5$ pfu
	2×10^7	5·7	3/3	
	2×10^6	9·3	3/3	
NYBH	2×10^8	3·7	3/3	$2{\cdot}9 \times 10^6$ pfu
	2×10^7	5·3	3/3	
	2×10^6	6·0	1/3	
Lister	2×10^8	5·7	6/6	$6{\cdot}3 \times 10^6$ pfu
	2×10^7	4·6	5/6	
	2×10^6	5·0	1/6	
EM 63	2×10^8	5·5	2/3	$1{\cdot}4 \times 10^7$ pfu
	2×10^7	5·0	2/3	
	2×10^6	13·0	1/3	
DIs	2×10^8	.	0/3	$\geqslant 6{\cdot}3 \times 10^8$ pfu
	2×10^7	.	0/3	
	2×10^6	.	0/3	
	TCID50			
Lc 16	2×10^8	.	0/3	$\geqslant 6{\cdot}3 \times 10^8$ TCID50
	2×10^7	.	0/3	
	2×10^6	.	0/3	

death or at moribund; most asymptomatic cases were sacrificed on the 14th day post-inoculation (p.i.) and some between the 5th and 7th day for histological examinations and for isolation of the virus.

Specimens were taken from the cervical and lumbar cord and the region of precentral gyrus for the virus isolation. Embryonated eggs were used for the isolation of the egg-grown virus strain and Vero cells for the Lc 16 strain. Portions of 0·1 ml of an appropriate dilution of a 10% homogenate of each specimen was inoculated on to the chorioallantoic membrane of 3–4 eggs or into five tubes of the Vero cell cultures.

The tissue specimens of the central nervous system and visceral organs were fixed with a 10% formalin solution and embedded in paraffin; sections were stained with haematoxylin and eosin. Additional sections were stained with Gallocyanine stain, Giemsa stain, Feulgen reagent or Luxol fast blue, when necessary. Tissue specimens of some animals were frozen in *n*-hexane cooled in dry ice-acetone for fluorescent antibody staining.

RESULTS

Symptoms appeared in the monkeys from 2 to 7 days p.i.; the monkeys became apathetic. In some animals, nystagmus and/or convulsion was observed. Most monkeys showing clinical symptoms died from 3 to 7 days p.i. The period from

Table II. *Virus recovery from the central nervous system*

Virus strain inoculated	Inoculum	Days p.i. dead or sacrificed	Clinical symptoms	Virus recovery from		
				Brain	Cervical cord	Lumbar cord
CV 1	2×10^7	4	+	5·3	6·2	3·8
		5	+	4·8	6·2	4·2
		6	+	2·0	2·9	1·2
	2×10^6	6	+	4·9	6·5	3·4
		10	+	3·9	5·2	2·6
		5	+	5·3	6·3	4·3
Lister	2×10^7	14	−	.	.	.
		5	+	4·9	6·0	4·1
		6	+	5·6	6·5	6·5
	2×10^6	5	+	4·4	5·8	3·2
		14	−	.	.	.
		17	+	0·9	.	.
EM 63	2×10^7	14	−	.	0·5	.
		5	+	6·3	6·5	5·1
		5	+	5·6	5·6	4·5
	2×10^6	14	−	4·2	.	.
		14	−	4·3	0·5	0·7
		13	+	.	1·8	.
Lc 16	2×10^8	7	−	.	.	.
		14	−	.	.	.
		14	−	.	.	.
	2×10^7	7	−	.	3·4	2·8
		14	−	.	.	.
		14	−	.	.	.
DIs	2×10^8	5	−	0·3	2·9	.
		14	±*	.	.	.
		14	±†	.	.	.
	2×10^7	7	−	.	.	.
		14	−	.	.	.
		14	−	.	.	.

* Fever, nystagmus, anorexia (5–7 days p.i.).
† Fever, anorexia, slight paralysis of the legs (6–8 days p.i.).

onset of illness to death was shorter in the monkeys inoculated with CV 1 and Ikeda strains than those inoculated with other strains.

The CV 1 and Ikeda strains were found to be more virulent, with an LD 50 of less than $6{\cdot}3 \times 10^5$ pfu. The virulence of the NYBH strain was slightly higher and that of the EM 63 strain slightly lower than that of the Lister strain. The DIs and Lc 16 strains were obviously of lower virulence than the other strains (Table I).

Viruses were isolated from the specimens of the succumbed monkeys. The virus titre was about 10^4 to 10^6 pfu/ml. The specimens taken from the asymptomatic monkeys inoculated with the Lister strain which were sacrificed on the 14th day did not contain detectable virus but those of monkeys inoculated with the EM 63 were positive in 3 out of 4 cases (Table II).

Histological findings of the inoculation sites were similar with all the strains tested, but severity of inflammation in the leptomeninx and choroid plexus differed. Dominant histological changes found in the central nervous system were inflammation in the leptomeninx and spinal nerve roots, choroid plexus and ependyma. On the 2nd to 7th day, the majority of the cells present were polymorphonuclear leucocytes; on the 14th day, lymphocytes, plasma cells, and lymphoid cells appeared to be dominant.

Congestion, swelling of capillary endothelium, perivascular oedema, and dilatation of perivascular spaces and the ventricles with unusual accumulation of cerebrospinal fluid were observed in most succumbed cases. Monkeys inoculated with the DIs and Lc 16 strains, and a symptomatic case inoculated with the other strains, showed only mild leptomeningitis with neither dilatation of the ventricles nor unusual accumulation of the fluid. On the contrary, monkeys inoculated with the CV 1 and Ikeda strains and those succumbed by inoculation of the Lister, EM 63, or NYBH strains, showed severe inflammation in the leptomeninx and choroid plexus together with haemorrhage in the parenchyma of the brain; the haemorrhagic reaction was more marked in cases inoculated with the Ikeda and CV 1 strains.

Softening of the brain with few cellular elements appeared perivascularly or in the subependymal layer of the ventricles, especially in the occipital lobe and temporal lobe. Degeneration of nerve cells was often observed in the succumbed monkeys; oedema with perivascular demyelinasation and spongy state was also observed in some cases. Perivenous microbial proliferation was rarely observed. Softening or degeneration of the nerve cells was not observed in monkeys inoculated with the DIs or Lc 16 strain (Table III).

Amyloid deposition was observed in the kidney, adrenal gland and heart muscles; proliferative changes and fibrinoid degeneration in arterial walls of the brain were also recognized in some cases later than 7 days p.i.

In the succumbed monkeys the viral antigen was shown by fluorescent antibody staining in leptomeninges, choroid plexus, ependyma, vascular walls and the surroundings of spinal nerve roots, but not in the parenchyma of the brain or spinal cord.

DISCUSSION

Experiments were reported, especially in the early 1930s, on intracerebral inoculation of monkeys with vaccinia virus to study postvaccinal encephalitis(**2,3,5,7,9**). The clinical symptoms and dominant histological findings described in these reports correspond fairly well to those in our results, but softening in the parenchyma was hardly mentioned in previous reports.

In succumbed cases there was severe inflammation of the leptomeninx and

Table III. *Summary of virus isolation and histological findings of central nervous system of the monkeys inoculated with CV 1, Lister and Lc 16 strains*

	Strain																	
	CV 1						Lister						Lc 16					
Inoculum ...	2×10^7			2×10^6			2×10^7			2×10^6			2×10^8			2×10^7		
Clinical symptoms	+	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	−	−
Days p.i.	4	5	6	6	10	5	14	5	6	5	14	17	7	14	14	7	14	14
Virus isolation	+++	+++	+++	+++	+++	+++	−	+++	+++	+++	−	+	−	−	−	++	−	−
Inclusion body	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Changes in Leptomeninx	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+	++	++	+	+	++	+	+
Choroid plexus	+++	+++	++	+++	++	++	+	+++	+++	++	+	++	+	+	+	−	−	−
Encephalomalacic alteration	+	−	−	+	++	−	++	++	++	−	−	−	−	−	−	−	−	−
Degeneration of nerve cells	−	−	−	−	+	−	+	+	+	−	−	+	−	−	−	−	−	−
Spongioid degeneration	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−
Haemorrhages in brain	++	−	−	+	−	++	−	+	+	+	−	−	+	−	−	−	−	−
Perivascular oedema	+	−	++	+	+	−	+	+	+	+	−	+	+	+	+	+	+	+

the choroid plexus due to infection with the virus, followed by disturbance of the circulation in the central nervous system. Softening or degeneration of nerve cells may not be a direct reaction of the virus multiplication in these tissues from the findings in immunofluorescence. The softening may probably have been provoked by the circulatory disturbance from its locality; the degeneration of nerve cells may have been induced by the same mechanisms. This does not necessarily exclude the possibility of the same effect by the viral components or by-products of infection.

These histological findings appear to be similar to the toxi-infectious changes described by De Vries(1). In addition to these findings, there are findings suggesting an antigen-antibody reaction, i.e. accumulation of round cells, amyloid deposition, and proliferative changes and fibrinoid degeneration in the arterial walls. These results may be available in analysing the pathogenesis of postvaccinal encephalopathy and encephalitis.

SUMMARY

The virulence of the EM 63, Lister and NYBH strains do not differ significantly. The CV 1 strain was regarded as an attenuated strain(4) but the results presented here showed as severe reaction in the central nervous system of monkey as that caused by the Ikeda strain. The DIs and LC 16 strains were obviously of lower virulence than the other strains.

The results suggest that the affinity or capacity of multiplication of the vaccinia virus in the meningen is different from that in the skin. It indicates a promise of developing an attenuated mutant with low neuropathogenicity and appropriate affinity to the skin.

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Discussion

NETTER (France) I think that the communication of Dr Hashizume is very important because it poses the question whether the test of the intracerebral inoculation of monkeys is to be considered as a genetic marker or can be used for evaluating the neurovirulence in man. I personally am not in favour of the second possibility because (1) the histopathology in monkeys is not the same as that in human postvaccinal encephalitis, (2) with another virus, such as rabies virus, the more it is adapted to the nervous system, as in the case of fixed rabies virus, the less it is pathogenic by the parenteral route, (3) in monkeys virus is found in the central nervous system, but this is quite exceptional in human postvaccinial encephalitis.

HASHIZUME (Japan) Regarding the 3rd point, in human cases dying after vaccination, vaccinia antigen was found in the meninges of the medulla with the fluorescent antibody technique.

MARENNIKOVA (USSR) I just want to ask you to repeat the dose you inoculated intracerebrally into each monkey. Was this dose the same for each strain?

HASHIZUME (Japan) The virus was purified by sucrose density-gradient centrifugation and the titre of each strain was adjusted to 2×10^8 pfu/ml and subsequently diluted tenfold serially so that 0·5 ml inoculum used per monkey respectively contained 10^8, 10^7 or 10^6 and in some cases 10^5 pfu.

LANE (USA) Just to follow up on Dr Netter's comment, I agree with him completely on what he is pointing out. We have no good laboratory markers or models for postvaccinial or indeed many of our other major complications. This means that we really have to have sooner or later some direct classic comparison trials of our promising new strains with traditional strains in man. These are going to be difficult for various reasons and I think that Dr Henderson's eradication programme is going to succeed before we come up with the better strains.

Session VI

GENERAL DISCUSSION

KAPLAN (UK) Let me reopen the discussion, which started after Dr Dekking's paper about vaccination of hospital staffs and other special populations at risk. In the Meschede disaster of 1970 several unnecessary deaths from smallpox happened because the authorities, fearing to damage by vaccination with potent vaccine, gave them unproved killed vaccine and allowed them to develop variola major with several subsequent deaths. Since smallpox so often surfaces in hospitals in Western Europe and is so often a hospital infection, we should take some stand on the topic.

DEKKING (The Netherlands) The choice is between non-vaccination and vaccination or pre-vaccination which gives good immunity on second vaccination. Even the CV-2 strain may be all right for this, because it will give a small basic protection in the hospital population which will be ready to be pushed immediately by a classical vaccine in case of smallpox. It is better to have a small tiny basic protection than no protection at all as is actually the situation in most European hospitals, with the exception of most Amsterdam hospitals where CV-2 has been applied and is applied every 2 years.

EHRENGUT (West Germany) I cannot agree with the comment of Dr Dekking. Our problem is the undiagnosed case of smallpox in the hospital. A vaccination after the third day of smallpox is problematic. This was the case in Meschede. The unvaccinated nurse was vaccinated immediately and died of smallpox. Therefore the hospital personnel should be vaccinated in advance with a conventional strain.

KAPLAN (UK) It is not so that Jennerian vaccination does not work at this stage.

EHRENGUT (West Germany) But it cannot stop the development of smallpox.

KAPLAN (UK) We can modify the smallpox and prevent people from dying unnecessarily.

STICKL (West Germany) An attenuated vaccine might be of use to protect unprotected people after smallpox contact. Animal trials with MVA and variola challenge suggest that this might be successful, not for full protection but to prevent severe illness to develop.

GISPEN (The Netherlands) I cannot see the reasons why hospital personnel would refuse classical smallpox vaccine and accept vaccination with attenuated vaccinia strains of which we have no indication that these are safer with regard to central nervous system complications.

DEKKING (The Netherlands) It is not so much the risk of encephalitis as well as the vaccination illness which makes not only the nurses themselves but also the

hospital administration refuse the vaccination. It is my experience that if you start vaccinating a hospital and you vaccinate a certain number of nurses, the others observe them closely. When they see that very little disease and scarring develops, then the others come and there is no problem, but if you vaccinate them with a classical vaccine and all nurses become ill you get much resistance in that peculiar hospital environment.

GISPEN (The Netherlands) I have been engaged in vaccinations in institutes and hospitals and all the arguments I have heard were centred around the central nervous system complications and not around smallpox vaccination disease.

LANE (USA) In our hospital vaccination programme we do not give any primary vaccinations. We leave unvaccinated patients alone and revaccinate only. The rate of complications is low, acceptance is high and apparently illness which causes loss of time from work is rare.

NEFF (USA) I would like to second what Dr Dekking said, that if you are dealing with hospitals vaccination does become a problem. We are at present trying to increase the immunization level at Johns Hopkins. On the part of the nurses there is resistance, and perhaps rightly so, in that they do not want many of the nurses vaccinated while working in the wards for fear of spreading vaccinia virus to some of the patients with immunological disorders, eczema or other dermatological conditions. A suggestion to solve this problem by vaccinating the nurses before they go on vacation met resistance from the unions, stating that the employees are not to go on vacation and be sick from the vaccination during the 2 weeks they are on vacation during that year.

I do not think that the CV-1 vaccine is an alternative to the current strains or that there really would be a good attenuated strain at this time, but I hope that we may continue to work on attenuated-strain candidates so that perhaps these could be used in the future for the primary immunization of individuals.

Right now, as you state, most of the hospital people are revaccinees. We have stopped vaccinating children now in the USA. If we do not eradicate smallpox in the next 10–15 years, these individuals will become primary vaccinees, and then you could be faced with a really serious problem of having to give a lot of primary vaccinations to people that are becoming hospital workers, nurses and so forth. I hope that we do not get to that point and that smallpox is eradicated throughout the world, but perhaps we should begin to plan gradually for the event that it might not be.

EDSALL (UK) Since I have personally had a severe reaction following a late revaccination, 25 years after the primary, I would like to know how many of such late revaccinations are for all practical purposes like a primary reaction.

LANE (USA) We have no hard data, but we feel that about 20 years after the last vaccination patients get primary-like Jennerian vesicles and marked oedema and erythema. They do not seem to get much fever, however.

VON MAGNUS (Denmark) We have the impression that females tend to have more severe reactions than do males. Do others have a similar experience?

LANE (USA) Again we have no data but it is also our impression, that older women have more serious local reactions to late revaccination than do males.

TAGAYA (Japan) This morning discussions were held concerning the induction of neutralizing antibody after the vaccination with inactivated virus. We talk sometimes much about humoral immunity and other times much about cellular immunity. The importance of cell-mediated immunity in the recovery from the primary vaccination has been established in infants with hypo- or agammaglobulinaemia without thymic dysfunction. The role of neutralizing antibody has been suggested but we still do not know all about the immunity against vaccinia or variola infections.

HENDERSON (WHO) I return to the antibody question. I quite agree that there are many factors involved in immunity and protection here, but I wonder how many virus vaccines there are that we have licensed in which a substantial proportion of people have no neutralizing antibody after administration. I cannot imagine licensing a measles vaccine while perhaps 30% of the people have no neutralizing antibody against measles after you give the vaccine, and similarly with polio and influenza. It troubles me a little bit that we would consider some sort of procedure here in which perhaps 30% of these people do not have neutralizing antibodies. Maybe it does not mean anything but I feel a little concerned in an endemic area of having my child done this way and not knowing what to expect when we have the actual challenge with variola virus.

Another topic that puzzles us a little at WHO is what criteria should be used to select suitable attenuated strains. It is my feeling that Dr Marennikova's studies deserve particular attention because she has taken several strains of which we have some knowledge of the relative reactigenicity in various ways and tried to correlate them with certain laboratory tests. Similarly Dr Hashizume's studies, reported today, were very interesting. I wonder if I may ask a couple of questions that bother me as I look at the data of certain strains. The CV-1 strain has been developed primarily to deal with eczema vaccinatum at a time when we had the feeling that this was a bigger problem than we now appreciate. This strain, according to Dr Dekking's comment, seems to have some encephalopathy associated with it, the rate of which, as Dr Gispen pointed out, is probably not very different from that of previous strains in use, and when at the same time we look at Dr Marennikova's and Dr Hashizume's studies, the CV-1 strain certainly does not look like an optimum candidate in terms of prevention of central nervous system disease. If this is what we really are trying to do, one wonders if one should not think about this a little bit in terms of trying to develop some reasonable markers based on a correlation with known strains or strains on which we have reasonable information regarding their pathogenicity and proceed from this starting-point.

DEKKING (The Netherlands) Nobody knows the cause of postvaccinial encephalitis. As far as I know there are no indications that strain differences are involved. The frequency in different countries and in the same country in different years is so variable that some other extraneous cause must be at work. We are now

witnessing, I hope, a gradual disappearance of this condition for which the extended use of the Elstree strain cannot be credited. It is perhaps comparable to what happened with the lethargic encephalitis, which appeared and disappeared in Europe in a 30-year period.

VON MAGNUS (Denmark) The problem of markers is very important in assuring that the strain in any lot of virus vaccine is identical with the parent strain. However, the use of markers for selecting a new virus strain as a candidate for a new virus vaccine is more difficult. The type 3 component in the oral poliovaccine has been considered as not quite satisfactory and efforts have therefore been made to find a new one. A type 3 was developed in Czechoslovakia and its ability to spread in the central nervous system of monkeys was found to be low, also when tested in other laboratories. This particular marker was found and is considered a very important one for oral poliovaccine virus and this strain was therefore tested in humans. However, paralytic type 3 polio cases occurred in relation to its use in children and this particular strain has therefore not had any further use for human vaccination.

HENDERSON (WHO) Dr Dekking posed a basic question asking whether there are differences or whether the various vaccinia strains are similar and all of this is an incidental finding. I think there may be differences and I have heard some who felt otherwise during this conference.

Dr Von Magnus raises a very difficult question and I quite agree that we have got to be very careful with laboratory markers, but somehow we must make some sort of selection when deciding to proceed with a large study in the field.

NEFF (USA) We must be careful not to mix apples with oranges. Certainly it is important to develop animal models to try to answer some of these questions. In the matter of encephalitis we must distinguish between infectious encephalitis and postinfectious encephalitis. The main problem in the field is postinfectious encephalitis, therefore we should work on animal models which duplicate the postinfectious encephalitis and within these animal models test various vaccine strains. But you cannot test for infectious encephalitis in animals and then expect to be able to make an analogy with postinfectious encephalitis in humans. It does not make sense.

KAPLAN (UK) I think the problem is that nobody as yet has come up with a decent animal model for postinfectious encephalitis.

TINT (USA) In view of the possible lack of correlation between the results of neurovirulence testing of vaccinia strains in animals and the incidence of postvaccinal complications in man, there may be the possibility of a direct human marker in some experimental data provided recently by Dr Tagaya. In a limited experiment in which the EEG's of postvaccinees were examined 10 days later, there was evidence of some abnormality, though transient, in 1 of 5 children vaccinated with the Ikeda strain and in 5 of 19 children vaccinated with Lister vaccine, whereas no abnormality was observed in 37 children vaccinated with CV-1 vaccine. This could be an approach to a human marker.

SESSION VII

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Chairman: Professor D. G. EVANS (UK)
Secretary: Dr A. C. HEKKER (The Netherlands)

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SESSION I: IMMUNITY AND SMALLPOX ERADICATION

Chairman: DR A. S. BENENSON

The introductory session of this meeting delineated the present status of the smallpox problem, the progress of the WHO eradication programme and the place of vaccine immunity in the thinking for the future.

Dr Henderson described the excellent progress of the eradication programme. This has been achieved not by vaccination alone but by the institution of active surveillance activities. None of the 30 endemic countries produced vaccine which met WHO standards in 1967; now more than two-thirds of the vaccine used in these areas are locally produced. Endemic disease is believed to be limited now to seven countries – four in Asia and three in Africa.

Dr Foege recounted the course of the co-ordinated regional campaign in the 20 countries of West Africa, which included the five countries with the highest smallpox incidence in the world in 1967 when the campaign started. Mass vaccination was combined with surveillance and epidemiological investigations of each case. This permitted selective epidemiological control to break the chain of transmission; 19 of the 20 countries became smallpox-free by October 1969 and the last case in the region was reported in May 1970. The surveillance system has reported 7 cases of human monkeypox in the last 2 years but no smallpox in the region in $2\frac{1}{2}$ years.

Dr Benenson reviewed the changes in the vaccine and vaccination techniques from a painful disease-producing measure forced on the public by determined health authorities to a procedure which has made it possible to apply effective vaccine of adequate potency to those segments of the populations most involved in transmission of smallpox. He warned against relaxation of the effort until the last pox virus had been removed from all deep freezers, in case resurgence of disease from an animal or remote hiding place occurs.

Professor Gispen instilled a note of great concern during his discussion of the immunology of monkeypox virus. He included data on three isolated strains – two from healthy cynomolgous kidney cells in Bilthoven (obtained from monkeys coming from Malaysia 52 days before) and one from a chimpanzee in the Congo which had been captured in a search for a monkeypox reservoir in an area where a human monkeypox case, but no smallpox, had occurred. These three isolates are indistinguishable by any laboratory test from variola virus. It is to be noted that human smallpox was not present in the area at the time and had not been present for some time.

Dr Perkins reviewed the sequence of events which led to the recent decision by the UK to abandon vaccination as a routine procedure. He indicated that

this decision was subject to re-evaluation should smallpox reappear. He hoped that if that occurred a safer vaccine would then be available. The discussion of this section leads to two recommendations:

(1) That *all* countries should have a well-developed and implemented programme for surveillance to detect any smallpox, and a plan for immediate control.

(2) That steps to develop completely safe vaccination should be actively pursued.

SESSION II: PRODUCTION OF SMALLPOX VACCINE

Chairman: DR P. FENJE

Preparation methods for large-scale production of potent and stable freeze-dried smallpox vaccine are well established. These methods, as recommended by the WHO, are simple enough to be easily adopted by laboratories lacking previous experience in the production of sophisticated biological substances. It seems that the present requirements of the WHO are more readily attainable with vaccine of calf origin than with vaccine produced in eggs. Although the latter has some advantages, particularly with regard to economics, the stability of vaccine lots produced in eggs is insufficiently consistent.

Purification of the seed virus by several cycles of differential centrifugation causes the elimination of interferon and probably of other inhibitors. Using a purified seed, the viral yield in the harvest can be almost doubled.

Treatment with Freon 113 followed by differential or zonal centrifugation results in a vaccine preparation essentially free of non-specific proteins. There seems to be a need for a single-dose vaccine container more practical than the presently available ones. The answer might be a drop of vaccine freeze-dried within a plastic applicator, held in a vacuum-sealed vial. Vaccination can be accomplished without reconstituting the dried vaccine.

Of practical importance might be a freeze-dried vaccine suspended in a high-viscosity silicone ointment.

This preparation appears to retain its potency for several months. Such vaccine can be used in multiple containers or in disposable single applicators.

Finally, the availability of an effective inactivated vaccine would be of advantage for that class of potential vaccinees at particular risk because of age or certain conditions. Studies using ethyl alcohol as an agent for both purification and inactivation seem to indicate that the immunizing antigen may be separated from sensitizing substances. More basic research is needed before recommendation for the use of such a vaccine could be made.

SESSION III: LABORATORY TESTING OF SMALLPOX VACCINE

Chairman: PROFESSOR W. HENNESSEN

The success already achieved by the WHO smallpox eradication programme clearly reflects the outstanding progress that has been made in vaccine production and quality control by participating laboratories throughout the world. The WHO programme can be expected to be continued with vaccines that meet the WHO requirements for Smallpox Vaccine.

It is probable that in the future, as the WHO programme progresses, the demand for smallpox vaccine will decrease. Such decreasing demand, together with the advance in technology in vaccine production, could be the starting-point for the development, in certain countries, of new types of vaccines (such as tissue-culture vaccines) which would be able to meet the laboratory requirements applicable to other live virus vaccines. It is important, however, that extensive tests for safety and efficacy in man should be made before any new vaccine is accepted for general use.

Techniques of potency testing employing various methods were presented and discussed. Results from many laboratories strongly indicated that the CAM potency test as recommended by the WHO is satisfactory for conventional vaccines, including highly purified products. When a reference preparation of the conventional type of vaccine is used, however, difficulties may occur in the CAM potency testing of tissue-culture vaccines and when the CAM test is compared with a tissue-culture potency test. The wording of the present WHO recommendation for potency does not stipulate that the expression of potency should be made in terms of a reference preparation. It was recommended that the potency requirement for smallpox vaccines should be framed so that the criterion of acceptability should be in terms of a reference preparation and preferably one equivalent to the International Reference Preparation.

Since manufacturers are now consistently producing vaccines with a very low content of micro-organisms, the requirement for total number of micro-organisms should be strengthened.

It was stressed that the WHO recommendations for smallpox vaccine should be met for all vaccines to be used in international programmes. National control authorities are, however, free to continue vaccine preparation according to their own regulations irrespective of WHO requirements.

For both liquid and freeze-dried vaccines promising procedures to improve stability were demonstrated. These improvements as far as freeze-dried vaccines are concerned may result in some complicated manufacturing processes.

The implication of the serological differences observed between intra- and extracellular forms of smallpox virus could not be fully evaluated. They do, however, deserve attention in relation to evaluation of efficacy. In addition,

further work should be directed towards investigating the effect of repeated passages on the biological properties of seed virus.

For practical reasons it seems necessary to clarify the intention of the heat-stability test described in the WHO Requirements. It is confirmed that by this test the stability shown should be such that the vaccine retains not less than one-tenth of its original virus content and in addition should fulfil the minimum requirement for pock count of 10^8 pfu/ml at the end of the incubation period.

SESSION IV: FIELD STUDIES

Chairman: PROFESSOR G. DICK

The field evaluation of new types of vaccines requires double-blind trials to test their protective effect and reactogenicity. It is unlikely that such trials are possible. However, a certain amount of evidence of their protective effect in the field may be obtained by testing the response to challenge with 'classical' strains. The local reactions which they produce may also be measured and protective antibody may be measured. Special attention should be paid to measuring the neutralizing antibody to extracellular virus, although it is likely that neutralizing antibody against intracellular virus and HI antibody are frequently concomitants of immunity.

Studies of administration of vaccine by the subcutaneous route may be associated with less local reaction and this method of immunization might be useful in the vaccination of individuals with atopic skin conditions. However, the importance of a scar as evidence of previous vaccination must be remembered.

The use of new systems for growing vaccine virus raises many problems: successful vaccination but as yet uncertain protection has been achieved by growing virus on diploid cells and primary rabbit kidney cells, etc. The reactivity of vaccines made from these cultured viruses requires further study. When these newer culture methods are used the possible presence of as yet undiscovered 'fellow traveller' agents must always be kept in mind.

SESSION V: COMPLICATIONS FOLLOWING VACCINATION

Chairman: Dr M. LANE

The excellent papers on vaccination complications are difficult to summarize. It is gratifying to see so much good information being gathered on this important subject, which was so long ignored. There are still problems of definition of cases and methods of study, problems which are always found in the study of rare events, particularly such poorly understood events as postvaccinal encepha-

litis. Rather than being concerned about the many minor differences between countries, which may in part be caused by differences in study methods, we should emphasize the many similarities.

The rates of major complications in several reporting countries seem to be decreasing. They are all considerably lower than rates found in older reports from Austria, the United Kingdom, and other areas. The previously common bacterial complications, particularly tetanus, have all but disappeared. The death-rate and case-fatality ratios from complications appear lower than in bygone days, perhaps because of modern treatment methods.

Undoubtedly the use of less-pathogenic strains of vaccinia such as the Elstree strain has contributed to the lowering of complication rates. On the other hand, data are available which suggest that there are other factors involved. Although there were no specific comments on the matter, possibly closer adherence to the accepted contraindications to vaccination has played a role. While there is disagreement about the optimal age for vaccination in non-endemic countries, at least each nation should be able to reduce its own toll of complications by vaccination at the age of its lowest rate of serious complications.

To consider contraindications to vaccination is a luxury which some endemic nations cannot yet afford. This, not because they lack the data, but rather because they still have smallpox. In nations with endemic smallpox the debate about complications rates is academic. The conference endorses the WHO expert committee's recommendations regarding the vaccination policy for endemic countries.

The low rate of serious vaccination complications means that direct comparison of two vaccine strains in a controlled trial, with these rates as the major variable, is impractical if not impossible. Those who are engaged in the development and evaluation of new strains and techniques of administration are faced with a staggering task in demonstrating decreased rates of serious complications. Yet until we have such data it is unwise to give up the present accepted and tested vaccines.

There is probably an irreducible minimum rate of complications, and some complications might occur with any vaccine, administered in any fashion, to any age-group. The rate of complications is only one factor in the determination of national vaccination policies. We should all spend some time and effort estimating our risks of smallpox importation, our capabilities for handling importations should they occur, and the background immunity produced by our current policies. Some Western nations have elected to stop routine childhood primary vaccination. Others have decided to continue. Policies based upon continuing routine vaccination perhaps before the very last smallpox case occurs seem dubious. However, there is no magic formula which can help us determine exactly when to de-emphasize this traditional public health technique.

SESSION VI: VACCINE DEVELOPMENT ESPECIALLY PERTAINING TO PREVENTION OF COMPLICATIONS

Chairman: Dr C. Kaplan

Section F concerned itself mainly with the problems of attenuated vaccines and the circumstances of their use. The most widely used attenuated vaccines have been those prepared from the Rivers CV 1 and CV 2 strains. There has been some uncertainty about the attenuation of these strains and about their efficacy. There are also no clear indications for their use; should they, for example, be used for the immunization of eczematous children and the delayed primary vaccination of adults at risk, or should they be used routinely for all vaccinations? There is also some doubt about which is the most suitable strain to adopt. Useful work has been reported on methods for identifying in the laboratory those strains likely to be least pathogenic in field use. A serious difficulty here is that there is no satisfactory animal model for the most dreaded (but not the most frequent) complication of vaccination – postvaccinal encephalitis. Among methods proposed for the reduction of risks of central nervous complications is the prior use of killed vaccinia virus antigen. This has been extensively used but it is impossible to decide rigorously on its effectiveness: clinical impression is the only readily available criterion. A coherent policy is needed for the use of attenuated vaccines; and also more work on the definition of candidate strains of virus. A sensible policy is also needed for the practice of vaccination in non-endemic countries so that those potentially at risk of exposure to variola virus may be protected.

AUTHOR INDEX

In italics: author(s) of papers

SUBJECT INDEX

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INTERNATIONAL MEETINGS
of the
Permanent Section of Microbiological Standardization

I. Première rencontre européenne de standardisation biologique

Lyon 1955 (22–25 juin) (Documents multicopiés)

Diphtheria Toxoid – Tetanus Toxoid – Tuberculins in Human Medicine – Tuberculin in Veterinary Medicine – Poliomyelitis Vaccine – Gammag lobulin.

II. Atti del secondo congresso internazionale di standardizzazione immuno-microbiologica

Roma 1956 (10–14 settembre) (Ed. Technica Graphica, Via Gallia 150, Roma, 1957)

Lactobacilli and Lactic Ferments – Pertussis Vaccine – Polio Vaccine – Smallpox Vaccine – Typhoid Vaccine.

III. Proceedings of the 3rd International Meeting for Biological Standardization

Opatijà 1957 (2–6 September) (Ed. Tiskara, Izdavačkog Zavoda, Jugoslavenske Akademije, 1958)

The Use of the Tissue Cultures *in vitro* in the Control of Sera and Vaccines – Influenza Vaccines – Combined Vaccines – Brucellosis Vaccines – Sterility Tests of Sera and Vaccines – Round Table Communications and Discussions – Tetanus – Typhoid.

IV. Proceedings of the 4th International Congress for Biological Standardization

Bruxelles 1958 (24–30 juillet) (Ed. Imprimerie de Charleroi, Charleroi, 1959)

Staphylococcal Vaccines – Pyrogens and Their Control – Control of Phage's Products – Control During the Production – Control of Vaccines in Veterinary Medicine – Vaccinations for Children – Tuberculin – Live Vaccines – Sterility – Documentation and Bibliography – Conclusions and Resolutions.

V. 5th International Meeting for Biological Standardization

Jerusalem 1959 (13–20 September) (The Weizmann Science Press of Israel, Jerusalem, 1960)

Standardization of Prophylactics and Diagnostics in Human Virus Infections – Standardization of Prophylactics in Zoonoses and Antropozoonoses Caused by Viruses – Inactivated Poliomyelitis Vaccines – Living Poliomyelitis Vaccines – Combined Vaccinations – General Problems. Precaution and Safety Tests – Standardization of Prophylactics and Diagnostics in Leptospirosis – Precipitation in Agar Gels – Standardization of Bacterial Prophylactics and Diagnostics – Antigens of Parasitic Origin – C-Reactive Protein – Passive Haemagglutination – Antivenomous Immune Sera – Conclusions.

VI. **Proceedings of the 6th International Congress for Microbiological Standardization**

Wiesbaden 1960 (5–10 September) (H. Hoffmann Verlag, Berlin-Zehlendorf, 1961)

Polio Vaccine, Oral – Combined Polio Vaccines – Measles – Germ Counting – Disinfection – Veterinary Matters.

VII. **Proceedings of the 7th International Congress for Microbiological Standardization**

London 1961 (28 August to 1 September) (E. and S. Livingstone Ltd., Edinburgh and London, 1962)

Biological Standardization – The Use of Standards – International Standards – Adventitious Agents in Tissue Cultures – Diagnostic Sera for Viruses – Sterility Testing – Toxicity Testing – Tuberculin Problems – Staphylococcal Antigens and Antibodies – BCG Vaccine – Field Investigation in Relation to Standardization – Antiviral Agents – New Antiviral Vaccines – Helminth Vaccines – Reports Participants.

VIII. **Proceedings of the 8th International Congress for Microbiological Standardization**

Bern 1962 (18–21 June). Vol. 1 of the Progress of Immunobiological Standardization (S. Karger, Publishers, Basel, 1964)

Opening Session – Standardization of the Antibiogram and its Reagents Used – Standardization of Diagnostic Methods in Rheumatic Diseases – Standardization of Reagents in Virology – Standardization of Microbial Antigens – Standardization of Sterility Tests (Round Table) – Control of Biological Products Performed by Official Laboratories (Round Table) – Standardization of Reagents in Allergy (Round Table) – Standardization of Enzymes in Immunology (Round Table, – Standardization of Methods of Disinfection (Round Table) – Problems Concerned with Live Poliomyelitis Virus Vaccine (Round Table) – Summary of the Subjects. Conclusions and Resolutions.

IX. **Proceedings of the 9th International Congress for Microbiological Standardization**

Lisbon 1964 (1–5 September). Vol. 2 of the Progress in Immunobiological Standardization (S. Karger, Publishers, Basel, 1964)

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X. **Proceedings of the 10th International Congress for Microbiological Standardization**

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XI. Proceedings of the 11th International Congress for Microbiological Standardization

Milan 1968 (16–19 September). Vol. 4 of the Progress in Immunobiological Standardization (S. Karger, Publishers, Basel, 1970)

Immunoglobulins: Biochemistry, Immunochemistry, Molecular Biology, Preparation, Control, Assays and Standardizations; Special Ig; Antilymphocytic Ig; Anti-D Ig – *Local Immunity*; Enterovaccines and Oral Immunization – *Immunochemistry of Enzymes* – *Free Papers*: Biochemistry, Pharmacology, Antivenom Sera, Immunology, Tuberculins, Toxoids, Bacteriology, Virology.

XII. Proceedings of the 12th International Congress for Microbiological Standardization

Annecy 1971 (20–24 September). Vol. 5 of the Progress in Immunobiological Standardization (S. Karger, Publishers, Basel, 1972)

Developments with Hepatitis – Developments with Marek's Disease – Developments with Virus Vaccines – Tests for Oncogenicity of Viruses – Anti-viral Agents – Virus Pneumonia Vaccines for Calves – Developments with Bacterial Vaccines.

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1st Symposium, Opatijà 1959:	International Symposium of Immunology. Edited by Dr D. Ikić, Tiskara Izdavačkog Zavoda, Jugoslavenske Akademije, Zagreb.
2nd Symposium, Opatijà 1960:	International Symposium of Microbiological Standardization. Edited by the Direction of the Institute of Immunology, Zagreb.
3rd Symposium, Lyon 1961:	Production et contrôle du vaccin buvable contre la poliomyélite (Sabin). Duplicated.
4th Symposium, Lyon 1962:	Symposium international de virologie vétérinaire: Pestes porcines et fièvre aphteuse. Edited by the Office International des Epizooties (OIE) and the Association Internationale des Sociétés de Microbiologie (AISM).
5th Symposium, Prague 1962:	Pertussis Immunization. Duplicated.
6th Symposium, Lyon 1962:	Vaccination antivariolique (Smallpox). Edited by the Institut Mérieux, Lyon.
7th Symposium, London 1963:	Méthodes consacrées aux épreuves de stérilité. Duplicated.
8th Symposium, Opatijà 1963:	The Characterization and Uses of Human Diploid Cell Strains. Edited by the Direction of the Institute of Immunology, Zagreb.
9th Symposium, London 1964:	Tuberculins. Duplicated.
10th Symposium, Paris 1964:	Désinfectants. Not published.
11th Symposium, Lyon 1964:	Antigènes et vaccins pour la sérologie et la prophylaxie de la rougeole et de la rubéole. Edited by the Institut Mérieux, Lyon.

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Vol. 24:	38th Symposium, San Francisco 1973:	Preservatives in Biological Products.
Vol. 25:	42nd Symposium, Lyon 1973:	The Efficacy of Poultry Disease Vaccines.

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MINUTES OF THE MEETINGS OF THE COMMITTEE ON HUMAN DIPLOID CELL STRAINS

(Permanent Section of Microbiological Standardization)

1st Meeting	Zagreb, Institute of Immunology, 12–13 October 1964 (Duplicated)
2nd Meeting	Geneva, Hôtel Métropole, 26–27 May 1965 (Duplicated)
3rd Meeting	Philadelphia, Wistar Institute of Anatomy and Biology, 18 May 1966 (Printed)
4th Meeting	London, National Institute of Medical Research (Hampstead Laboratories), 16 September 1967 (Printed)
5th Meeting	Philadelphia, The Wistar Institute, 27 November 1968 (Printed)
6th Meeting	New York, Albert Einstein College of Medicine, 30 October 1969 (Printed)
7th Meeting	Geneva, Institute of Hygiene, 14 September 1970 (Printed)
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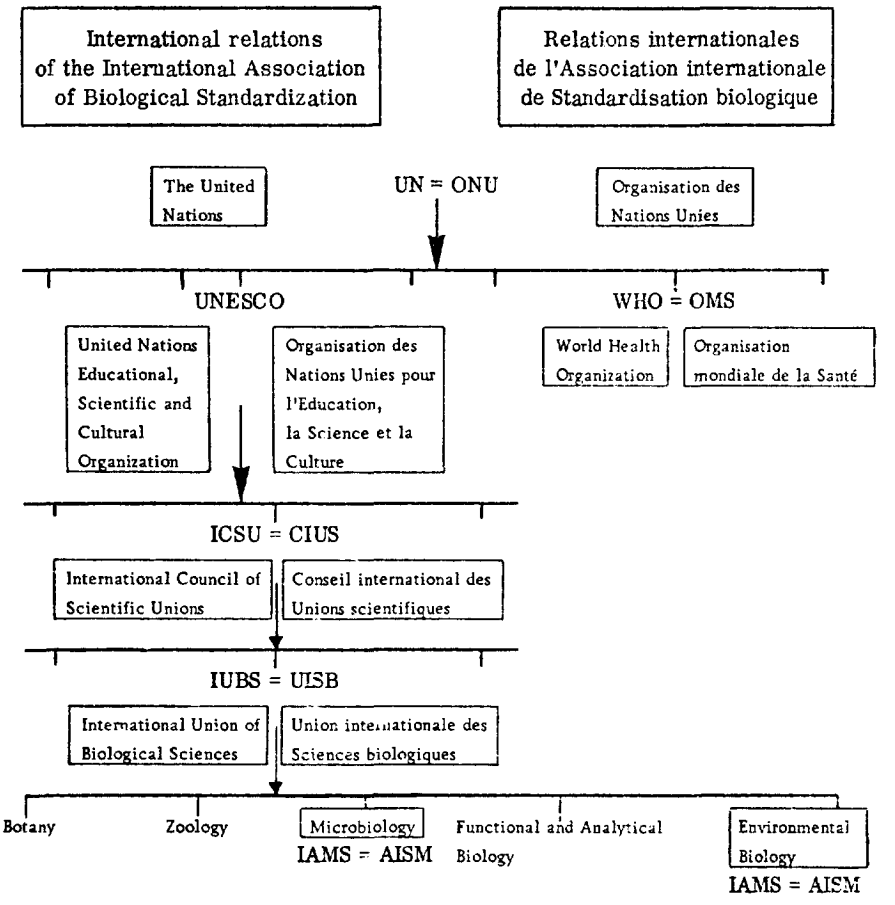
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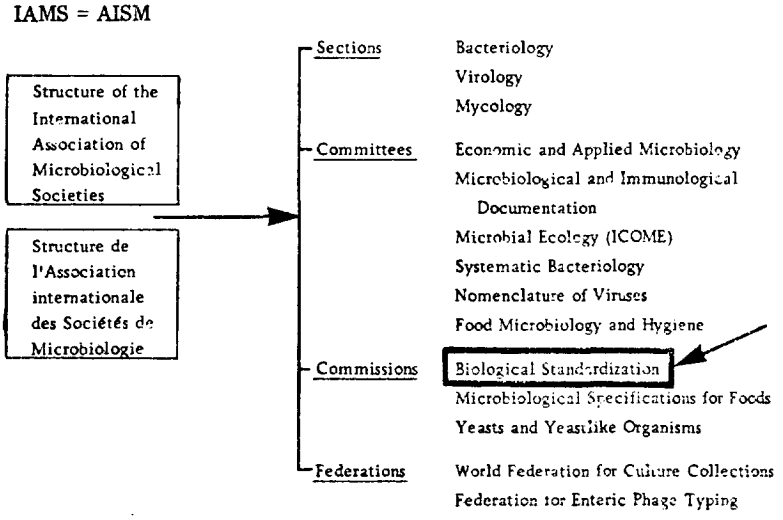
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1211 GENEVE 4
Suisse

The undersigned wishes to receive the following information:

Le soussigné désire recevoir les renseignements suivants:

.....

.....

.....

.....

The undersigned wishes to become a member of the International Association of Biological Standardization

Le soussigné désire devenir membre de l'Association Internationale de Standardisation Biologique

Name and address

Nom et adresse

.....

.....

.....

Official position

Titre set occupation

.....

.....

.....

Date:

.....

Signature:

.....

(Fees about US $20 = Sw. frs. 60)