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Inactivated smallpox vaccine. A comparison of inactivation methods

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SUMMARY

Vaccines were prepared from a single pool of high-titred vaccinia virus and inactivated by six methods, namely heat, formalin, hydroxylamine, β-propiolactone, ultraviolet irradiation, and visible light and methylene blue. Large doses of the vaccines were required to protect mice against intracerebral challenge. Differences in protection were not attributable to the method of their inactivation. The vaccines also induced similar degrees of skin immunity in rabbits which showed no severe dermal reactions when challenged with either homologous killed vaccine or live virus. The virus-neutralizing, haemagglutinin-inhibiting and complement fixing antibody responses to the vaccines differed; heat-inactivation preserved these antigens least well and β-propiolactone apparently the best. In both rabbits and mice there was little association between the different antibody responses to each vaccine or between the degrees of antibody response and the protection they induced. The relation of these findings to pox-virus immunity and the use of inactivated smallpox vaccine in man is discussed.

INTRODUCTION

There have been many attempts to produce an inactivated smallpox vaccine that would obviate the inherent hazards of conventional Jennerian vaccination. However, differences in preparation and in the assessment of their efficacy as antigens, either in man or animals, have produced many conflicting results (Janson, 1891; Parker & Rivers, 1936; Donally & Weil, 1940; Weil & Gall, 1940; Collier, McClean & Vallet, 1955; Herrlich, 1959; Beunders, Driessen & van den Hoek, 1960; Mahnel, 1960; Amies, 1961; von Epp, 1961; Lindenman & Buser, 1962; Kaplan, McClean & Vallet, 1962; Ramano Rao, 1962; Kaplan, Benson & Butler, 1965; Turner & Kaplan, 1965; McNeill, 1965; Madeley, 1968). Vaccines prepared from virus grown on different hosts have varied in antigen content, some were incompletely inactivated, and others may have lost immunogenicity as the result of the chemical or physical treatment used for inactivation. In the present study six commonly used methods of inactivation were used to make vaccines from a single batch of high titred virus. Immunogenicity was assessed by antibody response and skin immunity in rabbits, and by protection tests in mice. Immunization schedules and dosage were limited to those thought to be practicable when translated for use in man.

MATERIALS AND METHODS

Viruses

The Lister Institute strain of vaccinia virus was used for the preparation of vaccines. It was extracted from sheep dermal pulp in 0·015 M phosphate buffered saline at pH 7·0, purified by treatment with 'Arcton 113' (trifluorotrichloroethane I.C.I.) and differential centrifugation. A stock suspension containing 10^9 to 10^{10} pock-forming units (pk.f.u.)/ml. was stored at − 160° C. in 20 ml. volumes. Virus for the dermal challenge of rabbits was derived from chick chorioallantoic membranes (CAM) infected with the same strain. It was prepared and stored similarly, contained 3×10^9 pk.f.u./ml. and was bacteriologically sterile. The neurotropic strain of vaccinia virus (W.R.) used for intracerebral (i.c.) challenge of mice was a freeze-dried preparation of mouse brain. It was stored at 0–4° C.; when reconstituted and titrated in 16–20 g. mice it contained 10^4 LD50/0·02 ml.

Virus assay

0·1 ml. volumes of suitable dilutions of samples were inoculated on the CAM of 12-day chick embryos. Virus was estimated from the mean pock counts of 4–6 membranes after 48 hr. incubation. Titres are expressed in pk.f.u./ml.

Inactivation procedures

Dye and visible light. Virus suspensions were illuminated with a Philips Photolita bulb at 50 ft.c. intensity for 45 min. in the presence of methylene blue (3×10^{-5} M) (Turner & Kaplan, 1965).

Ultraviolet light. Virus suspensions were exposed for 30 min. in open Petri dishes 30 cm. distant from a UV tube (Philips 30 watt).

Heat. Virus sealed in suitable containers was completely immersed in a water bath at 60° C. for 40 min.

Formalin. Virus was stirred for 24 hr. at 18–20° C. in a dark container in the presence of formaldehyde (0·03 M) (B.D.H.A.R.) and glycine (0·04 M) (Gard, 1957).

Hydroxylamine. Hydroxylamine hydrochloride (Hopkins & Williams A.R.) was freshly prepared as a 2 M solution. The free acid was neutralized with NaOH before dilution to a final concentration of 0·1 M in the virus mixture. Inactivation was erratic and was only complete after long exposure (60–72 hr.) at 18–20° C. in the dark.

β-propiolactone (B.P.L.). Concentrations ranging between 1/250 and 1/500 were used, the mixture being held either at 0–4° C. or mechanically stirred for 24 hr. at 18° C. Free acid produced during the hydrolysis of the inactivator was neutralized with a few drops of Na_2CO_3 (0·5 M). The inactivation of most of the virus was rapid, but there were often traces of viable virus left that required more agent to ensure complete inactivation.

Preparation of vaccines

Stock virus was thawed, diluted fivefold, given approximately 30 sec. ultrasonic treatment to disperse aggregates and mixed in 20 ml. lots with an equal volume of

inactivator. Each mixture was exposed for the required time and temperature to effect just complete inactivation. The virus was sedimented from the mixture at 20,000 *g* for 30 min. and resuspended in the original volume of 0·015M phosphate buffered saline (pH 7·0). The suspensions were again treated ultrasonically, and tested for inactivation. Stock virus submitted to similar procedures in the absence of inactivator controlled the efficacy of virus recovery, and provided virus for comparison with inactivated virus. All the inactivated vaccines contained virus equivalent to $10^{8·7}$ to 10^9 pk.f.u./ml. They were stored at $-160°$ C. and thawed for immediate use, all unused material being discarded.

Tests for inactivation of vaccines

All the killed vaccines were submitted to the series of rigorous tests described by Madeley (1968), to determine whether viable or reactivable virus was still present. These were briefly

(1) Two successive chorioallantoic passages in eggs to detect virus survival or multiplicity reactivation.

(2) Inoculation on the chorioallantoic membrane in the presence of a known quantity of live virus to detect interference by large quantities of dead virus or the reactivation of dead by live virus.

(3) Intradermal inoculation into the shaved flanks of rabbits to detect dermal toxicity or viable virus.

In our hands, however, the intradermal inoculation of rabbits proved to be an unreliable test for complete inactivation. Some samples negative in rabbits yielded viable virus after two passages in eggs. A total volume of 1·0–2·0 ml. of each vaccine was tested. Vaccines were not used if any evidence of viable virus was obtained.

Immunogenicity of the vaccines

Mouse protection

Subcutaneous injection of large doses of most strains of vaccinia virus produces neither lesions nor illness in adult mice although transient infection occurs with live virus (Rosenau & Andervont, 1931; Briody, 1959; Turner, 1967). Male T.O. strain mice (Scientific Animal Service, Elstree) were used at an initial weight of 11–14 g. The methods of immunization and challenge are similar to those used in the NIH potency test for rabies vaccines (Seligmann, 1966). Three serial tenfold dilutions were prepared from each vaccine in dilute (0·015M) phosphate buffered saline (pH 7·0). Each dilution of vaccine was used to immunize a group of 10–20 mice; a similar group received undiluted vaccine. Two subcutaneous injections of 0·5 ml. were given at intervals of 1 week. Similar groups of mice were immunized with dilutions of live virus. One week after the second dose 5–10 mice from each group receiving the undiluted vaccine were killed and bled for antibody assay. The remaining mice were challenged intracerebrally with an estimated 5–50 LD50 of the neurotropic vaccinia strain W.R., which was titrated with each test in normal mice of the same batch. Mice dying within 48 hr. of challenge were discarded and the remainder were observed for 21 days. The 50% effective dose

(ED50) of each vaccine was calculated as the number of pock-forming units of virus, live or inactive, protecting 50% of the mice against the lethal challenge. Each vaccine was tested twice in this way.

Interferon in mice

Thirty mice were immunized with live virus; 10 were killed, bled and their brains removed. The remainder were challenged as above, and 24 hr. later blood and brains were taken from 10 of them. The 10 remaining mice, observed for 21 days, all survived. Control mice challenged in the same way were similarly sampled. Interferon was assayed in 10% (w/v) brain homogenates by the methods of Gifford (1963) and Subrahmanyan & Mims (1966). Virus-neutralizing (VN) antibody in sera and brain homogenates was estimated (see below).

Immunization of rabbits

New Zealand Red male rabbits weighing approximately 2 kg. were selected for skin areas free from pigmentation, hyperaemia and abnormal rates of hair growth. Each undiluted vaccine was tested in four rabbits in two intramuscular 1·0 ml. doses given 1 week apart. Four uninoculated rabbits served as controls. Four rabbits were vaccinated with live virus and housed in an area remote from those receiving either inactivated or no vaccine. The animals were bled before and 7 days after immunization and again 16 days after challenge.

Skin sensitivity

McNeill (1966) suggests that exaggerated skin reactivity may be induced by virus treated with particular inactivators. Accordingly two rabbits from each group were tested with 0·1 ml. intradermal doses of serial tenfold dilutions of killed virus 1 week after their second immunizing dose. In each case the immunizing and test material had been inactivated by the same method. To avoid any reactions due to sensitization by host components, the test material was made from virus grown in CAM and not in sheep. Two rabbits immunized with live virus were at the same time tested with a 1/10 dilution of all the variously inactivated preparations. Saline was used as a control. Photographic records as well as visual estimates of any dermal reactions were made after a few hours, and daily for 1 week. The animals were bled 16 days after the skin test, and challenged dermally with live virus.

Dermal challenge

One week after their second immunizing dose, 0·1 ml. of serial fivefold dilutions of live, bacteriologically sterile, CAM virus was applied to the scarified skin of the two remaining rabbits in each group. The animals were inspected daily, and the degree of skin resistance determined from readings on the fifth day, as in potency assays of smallpox vaccine. Photographic records as well as visual estimates of the lesions were made during the 14 days following challenge.

Antibody assays

Serum samples were inactivated at 56° C. for 45 min. and stored at -15° C.

Virus neutralizing antibody

This antibody was assayed as described by Turner & Kaplan (1965). Neutralizing potencies are expressed in international units (i.u.) calculated by reference to the potency of the international standard smallpox antiserum titrated with each assay. Each sample was assayed at least twice.

Haemagglutination inhibition

Vaccinia haemagglutinin was prepared from chorioallantoic membranes infected with Lister strain virus. They were extracted in saline and gross particles sedimented by low speed centrifugation. Haemagglutinin in the supernatant fluid was estimated with sensitive fowl cells. It was stored without further purification at -160° C. Dilutions of the sera were tested against eight agglutinating doses of haemagglutinin with 0·5 % fowl cells as indicator; unit volumes were 0·2 ml., and serum-saline was used as diluent (McCarthy & Helbert, 1960).

Complement fixation

Soluble antigen was prepared from a 10 % (w/v) suspension of sheep dermal pulp in phosphate buffered saline (pH 7·0). It was centrifuged at 20,000 *g* for 1 hr., and the supernatant fluid filtered through a millipore filter (pore size 0·22 μ). The filtrate was distributed in convenient volumes and stored at -15° C. When titrated against a hyperimmune sheep antivaccinial serum it had a titre of > 1/640 and was not anti-complementary when used in tests at a 1/20 dilution. The testing techniques and other reagents have been described (Madeley, 1968).

Gel precipitin tests

Undiluted 'soluble antigen' prepared for the complement fixation tests above was tested against undiluted and suitable dilutions of sera by gel diffusion in agar plates (Rondle & Dumbell, 1962). Hyperimmune sheep, normal rabbit and mouse serum were used as controls.

RESULTS

Immunogenicity in mice

The inactivated vaccines protected the mice only with doses 1000- to 10,000-fold larger than the protective dose of live virus. The dose response to all vaccines was linear and with one exception the slopes were parallel (Fig. 1). The small protective dose of live virus probably indicates the amount of Lister strain virus which establishes infection and viral multiplication in mice. The differences between the inactivated vaccines are not statistically significant.

Antibody response in mice

The antibody responses of mice are unrelated to protection (Table 1). No significant amounts of complement-fixing (CF) antibody were produced by any vaccine; as an index of an immune response to vaccinia in mice it appears to have little value. Neither was haemagglutinin-inhibiting (HI) antibody induced by most of the vaccines, it appeared irregularly after live virus and BPL-inactivated vaccine.

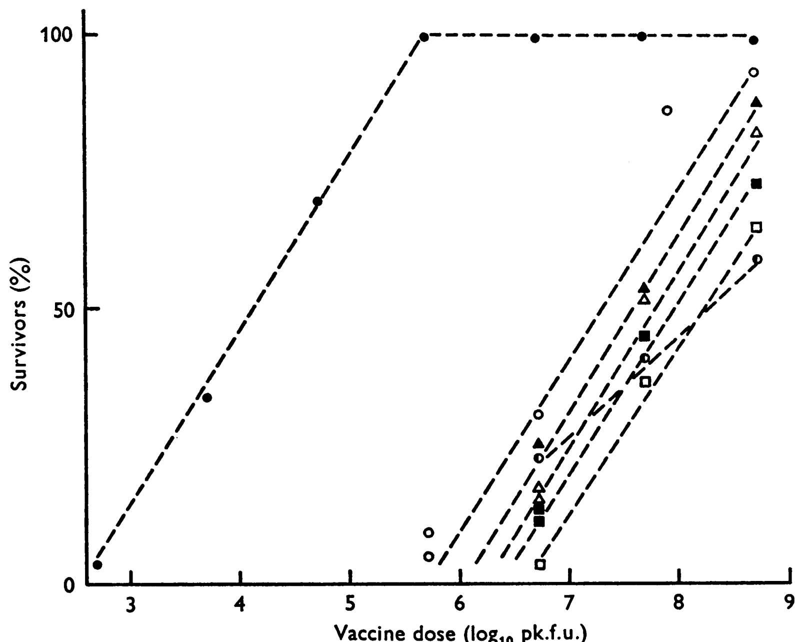

Fig. 1. Protection of mice with live and inactivated vaccines against intracerebral challenge (Av. 19 LD50). ●, Live vaccine; ○, vaccine inactivated by heat; ▲, formalin; △, β-propiolactone; ■, hydroxylamine; □, ultraviolet irradiation; ◑, photo-inactivation.

Table 1. *Response of mice to live and inactivated vaccines*

Method of inactivation of vaccine	Mean ED50 ($\log_{10}$ pk.f.u.)	Antibodies* at time of challenge		
		VN	HI	CF
Heat	7·4	0·3	< 5·0	< 20
β-Propiolactone	7·5	25	40	25
Formalin	7·7	7·2	< 5·0	< 20
Photodynamic	8·5	0·9	< 5·0	< 20
Ultraviolet	7·7	16·0	< 5·0	NT
Hydroxylamine	8·1	0·9	< 5·0	25
None (live virus)	3·9	1·2 to 90	< 5 to 40	< 20
No vaccine	—	< 0·05	< 5	< 20

* VN = Virus neutralization (International Units).
HI = Haemagglutinin inhibition (reciprocal titre).
CF = Complement fixation (reciprocal titre).
NT = Not tested.

Although the inactivated vaccines were equally protective, that inactivated by BPL induced comparatively high titres of virus-neutralizing (VN) and HI antibody, suggesting either that BPL preserved these antigens or that traces of live virus escaped detection in this vaccine (Kaplan, 1962). The ED50 of live virus makes this unlikely (Table 1) although vaccine treated more vigorously with this reagent gave an almost negative response.

Most of the vaccines induced VN antibodies, but in extremely variable amounts even in mice fully protected by live virus. None of the mouse sera produced precipitates in gel diffusion tests with the soluble antigen of vaccinia virus.

Interferon in mice

Although VN antibody was present in the blood of mice receiving protective doses of live virus none was detected in 10 % brain homogenates of the same mice either before or 24 hr. after intracerebral (i.c.) challenge. However, interferon-like substances were found in the brain homogenates after challenge, at a dilution of 1/18 in normal mice and 1/120 in vaccinated mice. In both cases the titres in pre-challenge samples were $< 1/5$. The results suggest that interferon is probably involved in protection.

Immunogenicity in rabbits

The challenge virus doses causing confluent or semiconfluent lesions on the skin of control animals produced only scanty or single lesions in animals immunized with most of the inactivated vaccines, indicating that they conferred a considerable degree of skin immunity, both 1 and 3 weeks after immunization (Table 2). Except for one rabbit receiving heat-inactivated vaccine, the responses to all inactivated vaccines were similar.

Lesions in immunized animals developed like those in the unimmunized up to the 5th day, but thereafter their evolution was accelerated. The individual pocks remained circumscribed, with moderate erythema surrounding them. There was no secondary spread, their mildly necrotic centres developed eschars by the 7th day, and healing preceded that of control animals by several days. In no case was there any evidence of the severe necrosis described in similar experiments by McNeill (1966). No lesions were produced in rabbits immunized with live virus.

Induction of dermal sensitivity

In rabbits injected with inactivated vaccines and tested intradermally with vaccine killed by the same method, the reactions were either trivial or absent. β-Propiolactone-inactivated vaccine induced early reactions in homologously immunized and unimmunized rabbits, but none of these developed into typical vaccinial lesions. Apart from this reaction, there were none with the other killed vaccines that could be associated with the use of a particular inactivator.

However, marked erythema and induration were produced by all the killed vaccines on the skins of rabbits previously immunized with live virus. They appeared after 24 hr. and persisted for almost 5 days before fading. Similar reactions are reported in man with killed vaccine administered to already vaccinated

individuals (Ehrengut, 1968). The reactions must be due to viral components in the test vaccine, since any host protein in the immunizing vaccine was different from that in the challenge vaccine.

Table 2. *Dermal response of individual rabbits immunized with live and inactivated vaccines*

Inactivation method for immunizing vaccine	Dose of live CAM virus (pk.f.u. by 10^{-3})			
	30	6	1·2	0·24
Heat	C	Sc	2	0
	3	2	0	0
	8	1	0	0
	2	0	0	0
β-Propiolactone	3	1	0	0
	2	0	0	0
	1	0	0	0
	1	0	0	0
Formalin	3	1	0	0
	1	0	1	0
	2	1	0	0
		Fur overgrown		
Photodynamic	3	1	0	0
	3	1	0	0
	6	2	0	0
	2	0	0	0
Ultraviolet	5	0	1	0
	1	0	0	0
	1	0	0	0
	2	0	0	0
Hydroxylamine	3	1	0	0
	2	0	0	0
	1	0	0	0
	1	0	0	0
None (live virus)	0	0	0	0
	0	0	0	0
		NT		
		NT		
No vaccine	C	Sc	1	0
	C	Sc	Sc	4
	C	C	3	1
	C	Sc	7	0

C, Sc = Confluent or semi-confluent lesions.
Numerals = Numbers of discrete pocks.
NT = Not tested.
The first two rabbits in each group challenged 1 week, the second two rabbits 3 weeks, after immunization.

Antibody responses

Antibodies were absent from all preimmunization sera. The virus-neutralizing antibody response to the killed vaccines was substantial, except after heat-treated

virus. The CF antibody response was moderate. HI antibody was usually absent (Table 3). HI antibody is considered to be a response to viral replication (Kaplan, 1962); with some anomalies, the present results confirm this, though large doses of inactivated vaccine can induce HI responses (Madeley, 1968).

There is little correlation between serum antibody concentration measured by the three methods; they are unrelated to the skin immunity induced in the same rabbits (Table 2), nor do they indicate the superiority of a particular inactivator, except perhaps BPL, in preserving the antigens that elicit them. The antibody

Table 3. *Antibody responses of individual rabbits to live and inactivated vaccines**

Inactivation method for immunizing vaccine	After immunization			After challenge†		
	VN‡	HI‡	CF‡	VN	HI	CF
Heat	0·7	< 10	40	1882	160	300
	11·0	< 10	40	8710	80	300
	1·3	< 10	< 10	9·6	< 10	30
	3·0	< 10	80	5·6	10	60
β-Propiolactone	50	< 10	100	3040	80	320
	1064	40	120	5548	320	180
	513	20	—	515	10	120
	355	< 10	80	427	20	80
Formalin	76	< 10	100	9772	160	180
	68	< 10	50	724	40	160
	32	< 10	40	80	< 10	60
	25	< 10	40	45	< 10	60
Photodynamic	21	< 10	40	1000	80	180
	69	< 10	60	760	10	120
	13	< 10	40	14	< 10	60
	3·2	< 10	30	4·6	< 10	40
Ultraviolet	69	40	40	5495	320	300
	87	10	30	1445	40	120
	29	20	80	275	20	140
	550	< 10	80	392	10	60
Hydroxylamine	115	< 10	< 10	1178	20	120
	38	< 10	30	119	< 10	60
	72	< 10	30	105	< 10	60
	19	< 10	30	101	< 10	50
None (live virus)	263	< 10	120	2970	160	240
	263	40	—	747	40	240
	1148	160	240	1995	40	240
	631	160	180	2098	20	240
No vaccine	< 0·05	< 10	< 10	903	160	240
	< 0·05	< 10	< 10	3706	160	320
	< 0·05	< 10	< 10	5·9	< 10	< 10
	< 0·05	< 10	< 10	0·6	< 10	< 10
Standard antibody	1000	80	300	1000	40	300

* The responses refer to the same rabbits as in Table 2.

† The first two rabbits in each group were challenged by dermal scarification with live virus, the second two by intradermal injection of homologous killed vaccine.

‡ See Table 1.

response obtained with BPL-inactivated virus was comparable with that of live virus. However consistent inactivation was difficult to achieve with BPL and, as described for mice, more vigorous treatment destroyed antigenicity.

Gel precipitation

Pooled sera from the groups of rabbits immunized with the inactivated vaccines gave no precipitates with soluble antigen in agar gel diffusion tests. A single broad line occurred with serum pools diluted 1/2 from rabbits immunized with live virus, and with 1/8 hyperimmune sheep serum. The production of visible precipitates thus requires serum of high antibody content. Such sera were obviously not produced by inactivated vaccines using the immunization schedule described here. The effect of any particular inactivation method on precipitating antigen cannot therefore be inferred from these results.

DISCUSSION

The kinetics of inactivation of viruses by heat, formalin, ultra-violet irradiation and dye-sensitized photoinactivation is already well documented (Woese, 1960; Gard, 1960; Taylor, 1960; Wallis & Melnick, 1965). β-Propiolactone reacts with all protein radicals and is commonly used to kill viruses for vaccines (Lo Grippo, 1960). It has seldom been used with vaccinia virus (Dostal, 1962). In our hands, inactivation was not easily controlled and requires further investigation.

Experimental vaccines inactivated by hydroxylamine have been prepared from fowl pest, influenza and foot and mouth disease viruses (Schäfer & Rott, 1962; Fellowes, 1966). Viruses differ considerably in their susceptibility to this reagent (Franklin & Wecker, 1959), and that of vaccinia virus has rarely been tested (Friedberger & Yamamoto, 1909; McNeill, 1965). We were unable to inactivate our virus suspensions completely at the concentration and exposure times recorded by McNeill. Hydroxylamine activity varies with electrolyte content and, in our hands, required approximately 0·1 M of the neutral salt for complete inactivation (Freese, Bautz-Freese & Bautz, 1961).

Heat, formalin and β-propiolactone can be considered as predominantly affecting the protein envelope of vaccinia virus. Ultraviolet inactivation, dye-sensitized photoinactivation and hydroxylamine inactivation of many viruses results principally from damage to the viral nucleic acid or nucleoprotein rather than the envelope protein, although it is doubtful whether this is always so (Turner & Kaplan, 1965; Kimes & Bussell, 1968). Absolute specificity of the site of action of the reagents is unlikely and the complex interrelated architecture of the pox viruses makes it doubtful that one site could be attacked without other modification of the whole structure (Gard, 1960). Pox virus inactivated by many of the commonly used methods can be reactivated after apparent loss of viability (Fenner, 1962; Abel, 1963; Kim & Sharp, 1967) and strains vary in resistance to some of the reagents (Sitnikov & Ghendon, 1968). Complete inactivation implies some irreversible change in nucleic acid since viral multiplication ultimately depends upon the integrity of this component. The antibody responses do not

illuminate these modes of action, apart from that of heat treatment, nor do they show the superiority of any particular inactivant.

Attempts to devise a mouse protection test for inactivated smallpox vaccine with a respiratory challenge were unsatisfactory (G. S. Turner, unpublished observations). The intracerebral challenge route used in the present experiments is fairly remote from natural infection and immunity to it is induced only by very large immunizing doses of killed vaccines (Bronson & Parker, 1944). The host factors that determine the resistance of mice against vaccinial infection are complex. Our observations offer no means of relating humoral antibody response to protection against intracerebral challenge. The suggestion that mechanisms other than orthodox specific immunity reactions are involved (Andrewes, Elford & Niven, 1948) are supported by observations of the role of interferon in vaccinia infection of mice (Finter, 1966). Our experiments suggest that intracerebral challenge may recall previously stimulated interferon (Baron, Buckler, Friedman & McCloskey, 1966); and since interferon is commonly elicited with inactivated virus it is likely that at least some of the protection afforded by the killed virus vaccines is due to this mechanism. Cellular immunity to vaccinia infection is demonstrable in mice (Hochstein-Mintzel, 1969) and is independent of antibody or interferon production. Its effects on intracerebrally injected virus are doubtful (Hirsch, Nahmias, Murphy & Kramer, 1968). We did not examine cell-mediated immune responses to our killed vaccines, but their importance in protection against pox virus infection is stressed by Boulter (1969).

We found no hypersensitivity to the killed vaccines in rabbits. The response to live virus after killed vaccine was accelerated, as in man on revaccination, but the severe necrotizing lesions described by Ramano Rao (1962) and McNeill (1966) were not observed. This may have been due to our use of bacteriologically sterile challenge virus or to the time interval between immunization and live vaccination. No such severe reactions have been reported when live vaccine follows killed smallpox vaccine in man (Beunders *et al.* 1960; Herrlich, 1959; Kaplan *et al.* 1965; Ehrengut, 1969). The element of hypersensitivity in the responses to both primary and secondary vaccination is well known (Allison, 1967) and indeed the response to revaccination is used as an index of immunity in man. Sensitivity and immunity to vaccinia virus however are not necessarily related apart from their common antigenic origin (Craigie & Wishart, 1933). Our results differ from those of Boulter (1969) in that we have always obtained strong evidence of dermal immunity in rabbits immunized with killed vaccine when challenged with homologous virus although it was unrelated to the magnitude of the humoral antibody responses. There is a similar absence of a relationship in man (von Epp, 1961; Beunders *et al.* 1960; Kaplan *et al.* 1965; Mannweiler & Geister, 1967), although the established value of hyperimmune globulin in the protection of smallpox contacts cannot be ignored (Kempe *et al.* 1961). It has been suggested that cellular mechanisms are more important in pox virus immunity than circulating antibodies (Boulter, 1969) and it is possible that the estimation of antibody is of little value in determining the immunogenicity of killed smallpox vaccine. Boulter also showed that antibody to killed vaccine differed qualitatively from that evoked by live virus. Nevertheless

rabbits immunized with killed vaccinia virus, while not protected from infection, survived challenge with lethal doses of rabbit pox (Boulter, Zwartouw & Titmuss, 1964; Madeley, 1968). We were also able to protect mice from similar challenge with adequate doses of killed vaccines.

Formalin-inactivated vaccine is used routinely in Germany for primary vaccination at 3 years or more. At the end of 1968 there had been some 250,000 recipients; dermal reaction and general symptoms on subsequent vaccination were little modified (A. Herrlich, personal communication) and though the incidence of post-vaccinial encephalitis was little affected its mortality was substantially decreased (Ehrengut, 1969; Rohde, 1968). The efficacy of inactivated vaccines in preventing subsequent vaccination accidents in man has yet to be adequately assessed in this country.

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