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The immunogenicity of heat-inactivated vaccinia virus in rabbits*

By C. R. MADELEY†
Department of Bacteriology, University of Liverpool

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Endemic smallpox is now confined to South East Asia and the tropical regions of Africa and South America, but may be casually imported into any other region. Travellers, and particularly air travellers, from endemic areas greatly increase the population who may be exposed to the disease, though risk to any one individual may occur very infrequently, if at all, and must be set against the chances of complications from vaccination with live virus. Even in the healthy these are not negligible, and are greatly increased in eczema and hypogammaglobulinaemia. An inactivated vaccine would be free from risks due to virus multiplication and would be a preferable alternative provided it could be shown to give adequate protection.

Several workers have reported experiments with animals and man using vaccinia inactivated by various means, and they have interpreted their results differently in the absence of any agreed criteria of immunity. Usually, immunity has been assessed by intradermal challenge with live virus, and, on this basis, some workers, e.g. Amies (1961) and RamanaRao (1962) in rabbits, and Kaplan, Benson & Butler (1965) in man, regarded the immunity produced by inactivated vaccinia as unsatisfactory.

Since only multiple intradermal doses of dermo-vaccinia virus will kill a rabbit, and even then not with certainty, workers attempting to assess the degree of immunity produced have been trying to measure degrees of skin immunity and the difficulties of doing this have been reviewed by McNeill (1966). In smallpox, where a viraemia is thought to play an important part in pathogenesis (Downie, 1965), circulating antibody may be the most important factor in deciding whether an infection develops, and the susceptibility of the skin may be of secondary importance. This assumes, however, that the antibody is efficient in neutralizing virus, and though development of neutralizing antibody has been reported by most workers when enough antigen has been given, the authors have said little about the nature of the antibody produced. Appleyard (1961) did note that the antibody elicited by rabbitpox soluble antigen in adjuvant was less efficient than that which appeared following natural infection with vaccinia, but other workers refer only to the titre.

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† Present address: Department of Medical Microbiology, St Thomas's Hospital Medical School, London, S.E. 1.

The ultimate aim of developing a vaccine for human use has led most workers to use a small number of injections. As a result the maximum response that a rabbit (or man) is capable of making to inactivated virus has not been completely assessed. Mahnel (1961) claimed that a single dose of high titre material elicited antibody, while McNeill (1965) obtained very variable results with single doses. It is difficult to see how useful estimates of immunity can be made unless enough antigen is given to ensure as uniformly high a response as can be obtained. Dosage schedules employing fewer injections or lower titre virus can then be compared with this as a yardstick.

Various inactivating agents have been used, but no one agent has emerged as clearly the best. Collier, McClean & Vallet (1955) obtained encouraging results in rabbits using ultra-violet-irradiated virus, both in terms of antibody produced and immunity to challenge, but results in a pilot study in man (Kaplan, McClean & Vallet, 1962; Kaplan, 1962; Kaplan *et al.* 1965) were regarded as unsatisfactory, in that the immunity to challenge was not reproduced though antibody was elicited. Amies (1961), RamanaRao (1962) and McNeill (1965) showed formalin-treated virus to be immunogenic in rabbits and Herrlich (1959; 1964) has used such a vaccine for pre-immunization before vaccination with live virus without, however, completely banishing complications.

In 1924, Nakagawa reported that rabbits inoculated with very large quantities of the closely related cowpox virus heated to 98° C. for 40 min. produced a good immunity to challenge with live virus. Later workers using heat as the inactivating agent have used either too much or too little. Parker & Rivers (1936) used 100° C. for 2 hr. and produced no immunity, whereas Gordon (1925) who used 55° C. for 30 min., and Kligler & Bernkopf (1935) who used 56° C. for 2 hr. produced good immunity probably resulting from surviving live virus. Woodroofe (1960) showed that virus stored for a week at 4° C. is inactivated more slowly than fresh virus, and the use of freshly prepared virus is essential if heat treatment is intended.

The present work was undertaken to explore immunity in rabbits to heated vaccinia virus, partly to follow up Nakagawa's work and partly because heating is a simple procedure which does not require the addition of any substances to the virus. It was intended to give enough antigen to ensure a maximal response, and the nature of the antibody produced was examined in detail. Finally, these results were related to the rabbits' ability to withstand intradermal challenge with vaccinia, cowpox or the more lethal rabbitpox, an infection similar in several respects to human smallpox (Bedson & Duckworth, 1963).

MATERIALS AND METHODS

Virus

The Lister Institute strain of vaccinia was used throughout to prepare vaccines. It was propagated on the backs of rabbits by lightly scarifying it into the shaved skin to produce confluent infection (Hoagland, Smadel & Rivers, 1940). Three days after infection the animals were stunned and exsanguinated, and the infected skin was removed. The dermal pulp from each infected area was scraped into 10–

15 ml. phosphate/phosphate buffer pH 7·2, ionic strength 0·01 μ, containing penicillin (100 u./ml.) and streptomycin (100 μg./ml.), and extracted by shaking with glass beads. The extract was clarified by low speed centrifugation and the virus was partially purified by centrifuging twice at 10,000 r.p.m. for 30 min. in the Type 40 rotor of the Spinco Model L. The international type strains of cowpox and variola major (Fenner & Burnet, 1957) and the Utrecht strain of rabbitpox (Jansen, 1941) were also used. The first was propagated exclusively on the backs of rabbits and the other two on the chorioallantoic membrane (CAM) of 12-day fertile hen's eggs. Virus was extracted and partially purified as already described. Virus for use in vaccines was used as an aqueous suspension. Stock virus was stored at $-20°$ C. as a 50 % suspension in glycerol.

Rabbits

New Zealand White and California rabbits were used to prepare virus. The vaccines were tested in 6-month-old Black and White Dutch rabbits weighing approximately 1 kg.

Infectivity titrations

Virus was diluted in phosphate buffered saline without magnesium or calcium (Dulbecco & Vogt, 1954) containing 10 % bacteriological nutrient broth, penicillin (100 u./ml.) and streptomycin (100 μg./ml.), and 0·1 ml. quantities were inoculated on the CAM of four or five eggs per dilution. End-point titres were calculated from mean pock counts obtained 2 days later with vaccinia or rabbitpox, and 3 days later with cowpox or variola.

Neutralization tests

These were done in eggs and were essentially the method of McCarthy & Downie (1948). Briefly, virus diluted to contain 50–100 pock-forming units (pfu) per 0·05 ml. was mixed with an equal volume of dilutions of serum which had been heated at 56° C. for $\frac{1}{2}$ hr. The mixture was allowed to react at room temperature (19–22° C.) for 1 hr. before 0·1 ml. was inoculated into four or five eggs per dilution. After incubation for 2 or 3 days as for infectivity titrations, pocks were counted, the results plotted on graph paper and the 50 % virus reduction titres obtained by eye. They were expressed as the $\log_{10}$ of the dilution, and are the mean of duplicate experiments.

Virus agglutination. Haemagglutination and haemagglutination-inhibition tests

The methods used were those of Craigie (1932) and McCarthy & Helbert (1960) respectively.

Complement-fixation tests

The method was essentially that of Downie & Macdonald (1950) using 3 in. $\times$ $\frac{1}{2}$ in. round-bottomed glass tubes, veronal buffer, and 2 % washed sheep cells (Burroughs Wellcome) sensitized with two minimal haemolytic doses (MHD) of rabbit haemolysin prepared in the laboratory. Unit (0·2 ml.) volumes of antigen, complement

(2 MHD/unit volume) and antiserum were added in that order, and fixation carried out at 37° C. for 1 hr. and 4° C. overnight before the addition of unit volume of sensitized cells. The mixture was incubated at 37° C. for 1 hr., and the results read after the remaining cells had settled. Appropriate antigen and antiserum controls were always included. The antigen used was 'soluble antigen', the supernatant of the first high speed centrifugation used in virus preparation.

Gel diffusion

The method used was similar to that of Rondle & Dumbell (1962). Ionagar (Oxoid), 1 % in distilled water, was autoclaved, and a 4 mm. layer was poured in a 3 in. Petri dish. Wells 9 mm. in diameter with centres 14 mm. apart were cut in the agar and the reagents were added undiluted and uninactivated. They were left to diffuse at room temperature in a humidified box for as long as necessary, and the lines of precipitate were photographed by dark-ground illumination when fully formed. It was found that the addition of buffer, salt or preservative to the agar did not improve the patterns, and often gave rise to granular, snow-like precipitates. No significant contamination with bacteria or fungi occurred. The antigens used were 'soluble antigens' as used for complement fixation.

Absorption experiments

The virus yield from the confluent growth of virus on the backs of twelve rabbits was pooled, partially purified by centrifugation and suspended in 10 ml. buffered distilled water. It was used to absorb small samples of antilive and antidead virus sera, either as live virus or after heating at 65° C. for 1 hr. The method used was as follows: To 1 ml. of unheated serum at 4° C. was added 0·5 ml. heated or live virus plus 0·05 ml. 9 % NaCl to make the mixture isotonic. The virus/serum mixture was allowed to react at 37° C. for 2 hr. and then at 4° C. overnight. It was clarified by centrifugation at 4500 r.p.m. in the bench centrifuge, and a further 0·5 ml. of virus suspension and 0·05 ml. of 9 % NaCl. added. It was allowed to react as before, followed by clarification and a final third absorption. After the final absorption the mixture was centrifuged at 20,000 r.p.m. for 1 hr. in the SW 39 rotor of the Spinco model L centrifuge, the supernatant was passed through Millipore membrane filters with an average pore diameter of 450 mμ and the filtrate tested, where appropriate, for residual live virus. None was found. These procedures resulted in dilution of the original serum 1:2·5, and this was included in calculating dilutions in subsequent tests.

IMMUNIZATION

Preparation of vaccines

Freshly prepared virus was always used, either uninactivated or inactivated by heat. Virus for inactivation was sealed in glass ampoules in approx. 5 ml. quantities, and heated by total immersion in a water-bath at 65·5 $\pm$ 0·5° C. for 1 hr. The virus was then removed and aggregates broken up by brisk pipetting with a pasteur pipette. The heated virus was used as an aqueous suspension for immunization and was stored at 4° C. during use. No adjuvants were used.

Testing of heated virus vaccines

Before use, each batch of vaccine was tested for residual live virus. The following tests were carried out:

(*a*) Quantities of 0·1 ml., containing the equivalent of 2×10^7 pfu, were inoculated undiluted on the CAM of five or six eggs. After 2 days the membranes were harvested aseptically, extracts were made and passed in further eggs. No pocks were ever seen on either first or second pass. Therefore, no evidence either of virus survival or of multiplicity reactivation was obtained.

(*b*) Small quantities of live virus, so as to give < 20 pocks per membrane, were mixed with the inactivated virus, and 0·1 ml. of the mixture was inoculated on the CAM of six to ten eggs. The expected number of pocks was always obtained showing that any live virus present was capable of expressing itself in the presence of large quantities of inactivated virus, and that the presence of some live virus did not reactivate detectable amounts of the heated virus.

(*c*) Serial tenfold dilutions of the vaccine were inoculated intradermally into the shaved backs of rabbits. Neither erythema nor oedema was seen. Further passage was not undertaken.

In addition to the above direct evidence, indirect evidence of complete inactivation was given by the qualitatively different response obtained in rabbits immunized with heated virus compared with those immunized with live virus.

Immunization schedules

Just before inactivation, preparations of virus were titrated in eggs, and estimates of the quantity of virus given in immunization are based on the pre-inactivation titre.

Groups of rabbits were divided into two subgroups. One subgroup was given heated virus and the other live virus. In all, thirteen rabbits were immunized with heated virus.

Heated virus. Initially, six intradermal and two intramuscular injections were given, containing the total equivalent of 10^9 pfu.

Three weeks later, a course of six intravenous injections was given, each containing the equivalent of 10^8 pfu in 0·5 ml. volumes. The injections were given twice weekly for 3 weeks, and the animals were bled from the marginal ear vein 1 week after the last injection.

Live virus. A similar schedule was followed except that a total of 300 pfu was given intradermally and no intramuscular injections were used. Three weeks later the same schedule was followed for intravenous injection and the rabbits were bled 1 week after the last injection.

Before immunization a sample of blood was taken from all rabbits, and the serum tested for pre-existing antibody by neutralization tests. None was found.

RESULTS

Antibody response

Following immunization with heated virus all the rabbits developed neutralizing, complement fixing (CF), haemagglutination inhibiting (HI) and precipitating antibodies. The titres of CF and HI antibodies were consistently lower than those in comparable antilive-virus sera. Typical examples are shown in Table 1 (complement fixation) and Table 2 (haemagglutination inhibition).

Vaccinial haemagglutinin has been shown to be separate from the virion, and is produced as a by-product of virus multiplication (Chu, 1948*a*). The inoculation of HA-free virus into rabbits results in the development of HI antibody (Chu, 1948*b*), and the failure to develop this antibody has been taken as evidence of complete inactivation of a vaccine virus (Kaplan, 1962). Therefore it was necessary to test the heated virus antigen used in immunization for the presence of haemagglutinin. The preparation tested caused detectable haemagglutination at 1/32, and the equivalent live virus haemagglutination titre was > 1/64. The procedures used to clarify the virus did not, therefore, remove all the haemagglutinin and, since it was stable at 65° C., HI antibody could have been and was developed.

Neutralization tests reflected similar differences in the neutralization of vaccinia virus (Table 3), but were less marked in neutralization of cowpox virus (Table 4). In all rabbits, however, a high titre ($> 10^4$) of antibody was found against both viruses. The un-neutralized fraction and the slope of the neutralization curves were similar to those found with sera made against live virus. The neutralization of smallpox virus by both types of sera was similar to that of vaccinia, though the titres were about one-tenth.

Gel diffusion tests in agar, however, showed clear qualitative differences between the two groups of sera. Plate 1, fig. 1, shows the comparison of four sera made against heated virus, nos. 108, 109, 110 and 111, with two sera made against live virus (72c and 130). Plate 1, fig. 2, shows the same sera compared with another antilive virus serum no. 55. It can be seen that in all cases the antidead virus sera give a single broad line which shows complete identity with one given by antilive virus sera. In addition, 72c and 130 show two other lines; one which is not given by any of the antidead virus sera and one which may be present in small quantity in 108 but not in the others. This is also apparent with serum 55, except that it shows two lines clearly not given by any of the antidead virus sera.

When a cowpox soluble antigen is used, the pattern shown in Pl. 1, fig. 3, is found. One (serum 72c) or two (serum 130) lines are given by the antilive virus sera, but no lines by the antidead virus sera. These sera are capable of neutralizing cowpox and the 'neutralizing' system would therefore appear not to be a precipitating one. However, Rondle & Dumbell (1962) showed that not all precipitating cowpox antigens are present in untreated soluble antigen. They reported a line pattern component '*f*' in vaccinia gel-diffusion patterns which could only be demonstrated in cowpox antigen following trypsin treatment. It is possible that trypsin might have released an antigen which reacted with the antidead virus sera and which might have been identified with '*f*'.

Table 1. *Complement-fixation test*

Serum no.	Made against	Antigen*	Dilutions	Serum dilutions 1/40	1/80	1/160	1/320	1/640
55	Live virus	L	1/40	++++†	++++	++++	+++	+
			1/80	++++	++++	+++	+++	+
		H	1/40	++++	+++±	++++	+	±
			1/80	++++	++++	+++	+	±
	Serum controls		.	−	−	.	.	.
65	Heated virus	L	1/40	++++	+++±	++	−	−
			1/80	++++	++++	+	−	−
		H	1/40	++++	+++	++	±	−
			1/80	++++	+++	+	−	−
	Serum controls		.	−	−	.	.	.

* Antigens: L, soluble antigen, supernatant of first high speed centrifugation in virus purification. H, soluble antigen, heated 65° C. for 1 hr.
† Results: ++++, complete fixation; +++, 75% fixation; ++, 50% fixation; +, 25% fixation; ± trace of fixation; −, no fixation, complete lysis.

Table 2. *Haemagglutination inhibition test*

Serum no.	Made against	Reciprocal serum dilutions 40	80	160	320	640	1,280	2,560	5,120	10,240	20,480
55	Live virus	−	−	−	−	−	−	−	±	++	+++
65	Heated virus	−	−	−	−	−	±	++	ND	ND	ND
25	Unimmunized control	+++	+++	+++	+++	+++	+++	+++	ND	ND	ND

Antigen controls Undiluted	Dilutions 1/2	1/4	1/8	1/16
+++	+	±	−	−

+++, complete haemagglutination; ++, approx. 50% haemagglutination; +, approx. 25% haemagglutination; ±, trace of haemagglutination; ND, not done.

A third group of rabbits was immunized partly to confirm the previous results, but also to obtain some information about the rise of antibody levels with successive doses. Before each intravenous injection a sample of blood was taken, with a final sample 1 week after the last injection. The antibody levels achieved were similar to those in previous groups, and a single line only was given in gel diffusion. Control rabbits receiving live virus developed almost maximum titres of neutral-

Table 3. *Vaccinia neutralizing antibody titres*

Antilive virus		Antidead virus	
Serum no.	Titre*	Serum no.	Titres*
112	4·5	108	4·8
114	4·9	109	4·7
115	4·7	110	4·4
117	4·8	111	4·3
128	5·7	113	4·6
129	4·8	119	4·3
130	4·8	131	4·8
Mean	4·9	Mean	4·6

* Log_{10} reciprocal of 50 % end-point titre.

Table 4. *Cowpox neutralizing antibody titres*

Antilive virus		Antidead virus	
Serum no.	Titres*	Serum no.	Titres*
112	4·1	108	4·8
114	4·5	109	4·5
115	3·9	110	4·1
117	4·0	111	4·4
128	4·5	113	4·4
129	4·2	119	3·9
130	4·5	131	3·7
Mean	4·2	Mean	4·2

* Log_{10} reciprocal of 50 % end-point titre.

izing and haemagglutination inhibiting antibodies after the intradermal injections alone. Precipitating antibodies in gel diffusion, however, did not reach a maximum number of lines until after two intravenous injections. In contrast, development of antibody to heated virus was more gradual with significant levels of neutralizing antibody appearing only after the second intravenous injection. The one precipitation line also appeared at the same time. Haemagglutination inhibiting antibody appeared more gradually, rising to a maximum only after five or six intravenous injections. Representative results from one rabbit in each vaccine group are shown in Fig. 4. It should be noted that all antibodies had reached a plateau of response by the time the last injection had been given.

The absorbed sera were tested for residual antibody activity. Absorption of the serum made against heated virus, using either heated or live virus, removed all detectable antibody activity, and, in gel diffusion, the preparation containing live virus as the absorbant showed *antigen* activity. In contrast, the serum made against live virus and absorbed with heated virus showed only slight diminution of activity

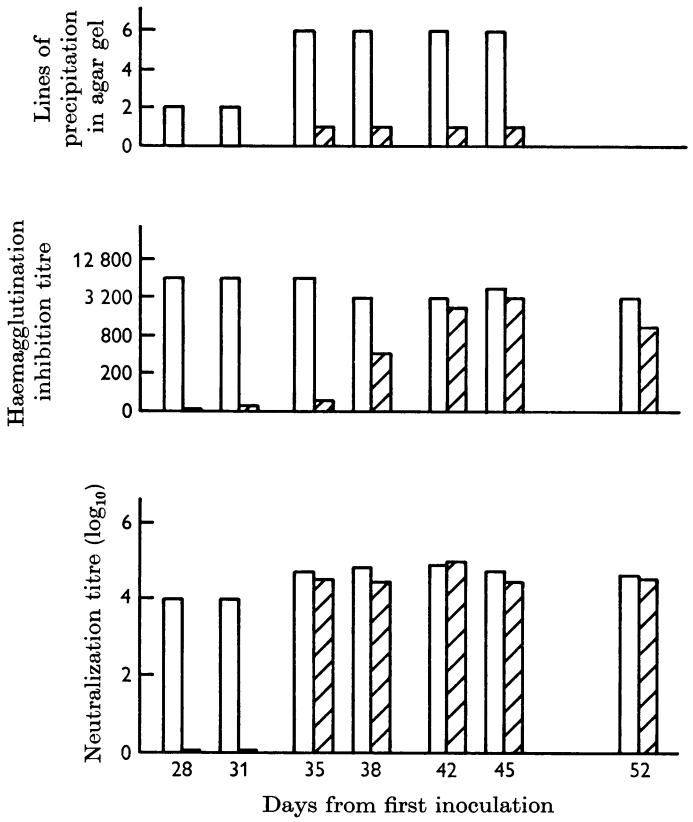

Fig. 4. Development of neutralizing, haemagglutinating and gel diffusing antibodies in two rabbits immunized with heated and live virus respectively. Rabbit 141 (□) was immunized with live virus and rabbit 144 ▨ with heated virus. Each histogram represents the titre of the antibody in a sample taken immediately before an intravenous injection of virus, the seventh sample (day 52) being taken one week after the last injection. See 'Immunization' in the text for details of injection schedules.

in neutralization, complement-fixation or virus-agglutination tests when unheated virus or viral antigen was used. In complement-fixation tests using heated antigen, only trace activity was detected and the absorbed serum failed to agglutinate heated virus at the lowest dilution tested (1 in 20). A summary of the results is given in Table 5. The neutralization results were confirmed using a different pair of sera and absorbing as before.

Table 5. *Summary of serological tests using absorbed sera*

	Serum no....	55			65		
	Made against...	Live virus			Heated virus		
Test	Absorbed with*...	Nil	L	H	Nil	L	H
Complement fixation	Live antigen	1/480	1/120†	1/240	1/160	< 1/40	< 1/40
	Heated antigen	1/240	1/60†	< 1/40	1/160	< 1/40	< 1/40
Virus agglutination	Live virus	1/160	< 1/20	1/80	1/80	< 1/20	< 1/20
	Heated virus	1/160	< 1/20	< 1/20	1/80	< 1/20	< 1/20
Neutralization‡	Vaccinia	4·5	< 1·0	4·0	4·3	< 1·0	< 1·0
	Cowpox	3·6	< 1·5	< 1·5	3·9	< 1·5	< 1·5
	Variola	4·2	< 1·5	3·7	4·1	< 1·5	< 1·5
	Serum no....	54			64		
	Made against...	Live virus			Heated virus		
Test	Absorbed with*...	Nil	L	H	Nil	L	H
Complement fixation	Live antigen Heated antigen	ND	ND	ND	ND	ND	ND
Virus agglutination	Live virus Heated virus	ND	ND	ND	ND	ND	ND
Neutralization‡	Vaccinia	4·7	1·5	4·6	3·9	< 1·5	< 2·0
	Cowpox	3·5	< 1·5	< 1·5	3·7	< 1·5	< 1·5
	Variola	4·6	< 1·5	4·2	4·2	< 1·5	< 1·5

L, absorbed with live vaccinia virus. H, absorbed with vaccinia virus heated to 65° C. for 1 hr.
† This serum was anticomplementary + + + + at 1/40.
‡ Log_{10} reciprocal of 50 % end-point titres.
ND, not done.

Challenge experiments

These were designed to test the immunity of the rabbits to challenge with live virus. The viruses used in some experiments were vaccinia and cowpox, both relatively non-lethal for the rabbit. In others rabbitpox virus, which is lethal for rabbits, was used.

Challenge with vaccinia and cowpox

The rabbits were challenged intradermally by graded $\log_{10}$ doses of live vaccinia virus into one shaved flank and cowpox virus into the other, the dose of virus used being confirmed in eggs. Equivalent doses of heated virus were given at the same time. The rabbits were examined on the fourth and seventh days after inoculation and the maximum size of any lesions appearing was recorded. Normal rabbits and rabbits immunized with live virus were included as controls.

Table 6. *Challenge with vaccinia of rabbits immunized with heated virus*

Dose in pfu...	1	10	10^2	10^3	10^4
Rabbit no.					
144	−	−	−	±	+ +N
145	−	−	±	+	+
	Controls				
(*a*) Normal rabbits					
156*	−	+	+N	+	+ +N
158*	−	+N	+N	+N	+ +N
(*b*) Immunized with live virus					
140	−	−	−	−	−
141	−	−	−	−	−

± Small papule.
\+ Lesion not more than 9 mm. in diameter.
\+ + Lesion not more than 19 mm. in diameter.
N Lesion showing necrosis.
* Secondary lesions present at 7 days.

The results of challenge of one group of rabbits with vaccinia are shown in Table 6. It can be seen that the immunity exhibited by rabbits 144 and 145 falls somewhere between the complete immunity, to the doses used, shown by the rabbits immunized with live virus and the susceptibility of the normal controls. Some lesions developed but only one rabbit (144) showed necrosis, and that only in one lesion. Necrosis was a common feature of the lesions in the normal controls. In addition, the normal controls showed secondary spread at 7 days and this was not seen in rabbits immunized with heated or live virus.

The lesions due to cowpox were very similar in extent and appearance though necrosis was more common, a feature of cowpox infection in the rabbit. The heated virus did not cause lesions on any rabbit, so that it is unlikely that hypersensitivity played a significant part in the development of the lesions due to live virus. Challenge of another group of rabbits on another occasion showed the same features.

Rechallenge with vaccinia and cowpox

These rabbits were re-challenged 1 week after complete healing of the first lesions, 35 days after the first challenge. Two fresh normal controls were included. The purpose of this rechallenge was to discover whether challenge with live virus had produced an improved immunity comparable to that produced by live virus alone. The results are shown in Table 7. Again, no lesions were produced at the site of injection of heated virus. Slightly smaller doses of virus were used in this challenge in an attempt to increase the sensitivity of the test.

Table 7. *Rechallenge with vaccinia of rabbits immunized with heated virus*

Dose in pfu...	10^{-1}	1	10	10^2	10^3
Rabbit no.					
144	−	−	−	+	++
145	−	−	−	±	+
			Controls		
(*a*) Normal rabbits					
150	−	−	++N	++N	++N
159	−	+	+	++	++N
(*b*) Control rabbits from first challenge					
156	−	−	−	−	±
158	−	−	−	−	−
(*c*) Immunized with live virus					
140	−	−	−	−	±
141	−	−	−	−	−

± Small papule.
\+ Lesion not more than 9 mm. in diameter.
++ Lesion not more than 19 min. in diameter.
N Lesion showing necrosis.

Comparison of Table 7 with Table 6 shows the following:

(*a*) The control rabbits of the first challenge (156 and 158) and the rabbits immunized with live virus show virtually complete immunity to the doses used.

(*b*) The new control rabbits show the same susceptibility as before.

(*c*) The rabbits immunized with heated virus show a similar susceptibility to rechallenge as they did to initial challenge, and had developed no increase in resistance. These results were confirmed in another group of rabbits.

Serum samples from these challenged rabbits were then examined by gel diffusion. Serum was obtained from the rabbits after immunization and before challenge (serum *a*), after challenge (serum *b*) and after rechallenge (serum *c*). At the same times sera were obtained from controls which were immunized but not challenged. The results of testing these sera are shown in Pl. 2, figs. 5–8. Rabbits 140 and 141 were immunized with live virus and were challenged twice, 144 and 145 were immunized with heated virus and were challenged twice. Rabbits 142 and 146 were immunized with live and heated virus respectively but were not challenged, and 156 and 158 were normal unimmunized rabbits which were challenged twice. The '*a*' sera from immunized rabbits gave the same appearance as found previously, the live virus sera showing 3 or more lines not present in the

heated virus sera. The post-challenge sera from the rabbits immunized with live virus showed no new lines, as expected. The same sera from rabbits immunized with heated virus showed the addition of a single faint line or zone after challenge. A comparison with vaccinia and cowpox soluble antigens suggested that this line was due to an antigen from the cowpox used in the challenge, rather than vaccinia. The complete vaccinia pattern was not developed—in contrast to the normal controls which developed 4 or 5 lines after the first challenge.

Challenge with rabbitpox virus

The purpose of this experiment was to examine whether the rabbits immunized with heated virus, though not immune to intradermal challenge, would yet possess enough immunity to protect them from a lethal virus. The lethal dose of

Table 8. *Challenge with rabbitpox*

Group	Immunized with	Lesions	Viraemia	Secondary spread	Temperature > 103° F.	Died
I	Heated virus	3/6*†	0/6	0/6	4/6	0/6
II	Live virus	0/6	0/6	0/6	0/6	0/6
III	None	6/6	6/6	6/6	6/6	5/6

* Figures in the table refer to the number of rabbits exhibiting the feature over the number in which it was sought.

† Maximum size of lesions were: rabbit 109, 58 mm.; rabbit 113, 15 mm. and rabbit 119, 8 mm. Doses received were: 109, 20 pfu; 113, 200 pfu; 119, 20 pfu.

vaccinia or cowpox viruses in a single intradermal injection is very large, and probably infinite, while that of rabbitpox is small, 0·1–1·0 pfu (Bedson & Duckworth, 1963), and it was felt that challenge with this virus would be a test of life-protecting immunity.

Three groups of six rabbits were used, six immunized with heated virus, six with live virus and six were unimmunized controls. It was decided to challenge the rabbits by intradermal injection rather than intranasal instillation because the dose can be more accurately controlled and infection can be observed more directly. Each group of six rabbits was divided into three subgroups of two, and each subgroup given a different dose of rabbitpox virus to discover whether the degree of immunity could be related to the dose of challenge virus. The doses used were 2, 20 and 200 pfu and were given as a single intradermal injection of 0·1 ml. into the shaved flank. The rabbits were observed daily for 2 weeks, and thereafter as necessary. Where lesions were observed, samples of blood were taken, prevented from coagulating with heparin, and tested for viraemia by inoculation undiluted into duplicate tube tissue cultures of RK 13 cells. The results are summarized in Table 8, in which the following can be seen:

1. No clear relationship between dose of virus and size of lesion was noted, though the only surviving control rabbit did receive the smallest dose.

2. No lesions developed in the rabbits immunized with live virus, though lesions were seen in three rabbits immunized with heated virus and one of them reached

more than 5 cm. in diameter, with marked necrosis. In none of the rabbits, however, was a viraemia detected on the ten occasions when it was sought.

3. In the unimmunized control rabbits, all developed lesions and five out of six died.

4. A viraemia was detected in all control rabbits and all of them developed secondary lesions. No secondaries were seen in either group of immunized rabbits.

5. Though the rabbits immunized with heated virus which developed lesions also developed a pyrexia, they were neither seriously ill nor anorexic.

DISCUSSION

The experiments reported in this paper were intended to establish the nature of the response of rabbits to inactivated virus, fulfilling the criteria of complete killing of the virus and administration of enough antigen to elicit maximum titres of antibody. They were not intended primarily as a pilot study of possible vaccines for human use, though this was the underlying purpose. In previously published work, rigorous proof of complete inactivation, including loss of the ability to be reactivated, has not been attempted. In the present work, no evidence of residual live virus in the heated vaccines was obtained, nor was there any evidence that the virus was capable of reactivation, either by live virus, in multiplicity reactivation or on passage. It was also shown that any live virus remaining would have been able to express itself amongst the dead. Heated virus has been shown to interfere with the replication of live virus but the ability to do so is lost in 30 min. at 60° C. (Galasso & Sharp, 1963). It might be expected that heating at 65° C. for 60 min. would also destroy the capacity to interfere. Further, since the response to heated virus was qualitatively different from that to live virus, it is unlikely that a significant quantity of live virus could have survived heating. It is probable then, that the responses were due only to the administration of heated virus, incapable of multiplication.

The experiments on dose requirements for maximum antibody production showed that enough antigen had been given to elicit a maximum response with the dosage schedule used. Neutralizing, haemagglutination-inhibiting and precipitating antibodies had reached a plateau of response, in which their titres were comparable to those produced by live virus. The antibody produced appeared to be similar to that produced by stimulation with live virus. Neutralization tests gave a similar pattern in terms of resistant fraction and the abruptness with which the end-point was reached. This is in contrast to the antibody response elicited by Appleyard (1961) using 'soluble antigen' in adjuvant, where the end-point was approached gradually with a progressive increase in the resistant fraction of unneutralized virus. In neutralization, complement-fixation, haemagglutination-inhibition and agglutination tests the antibody behaved in the same way as that to live virus. It was only in gel diffusion that qualitative differences were seen.

Here, the antidead virus sera gave only one line of precipitation with soluble antigen, three or four lines fewer than antilive virus sera. What these missing lines represent is not clear; they may be either antibodies to heat labile components of the virus or internal components not released with failure of the virus to replicate.

or they may be antibodies to by-products of virus growth, including enzyme systems. No information was obtained to distinguish between these hypotheses, but these missing antibodies do not appear to be the sole mediators of any of the serological activities measured in neutralization, complement-fixation, virus-agglutination or haemagglutination-inhibition tests. It is possible that the neutralization reaction does not precipitate and the results using cowpox antigen could support this. More information relevant to this point could be provided by using cowpox antigens treated with trypsin, etc.

The results of neutralization tests using absorbed sera showed that virus could be neutralized through heat-labile and heat-stable antigens. Sera made against live virus and absorbed with heated virus can contain no antibodies present in sera made against dead virus, particularly if all the antibody activity in antidead virus sera can be absorbed out with dead virus. Therefore, in the poxvirus system, there is no unique neutralizing antigen or antibody.

The precise mechanism of poxvirus neutralization has not yet been fully elucidated. Dales & Kajioka (1964), using the electron microscope, have shown that neutralized vaccinia is adsorbed to L cells and viropexis follows. Once within the cell the neutralized virus is gradually degraded without uncoating. They used live virus and antiserum made against it; whether antisera to heated virus would neutralize in the same way is not known, and this point might be further examined. However, these results tend to support the view that neutralization is due to antibody on the surface of the virion interfering with its replication in a relatively non-specific way.

Absorption of live virus antisera with heated virus did not reduce their ability to neutralize vaccinia but removed all detectable cowpox neutralizing antibody. This difference between the two viruses is interesting. It has been examined further and will be reported elsewhere.

The experiments on the dose required to produce a maximum response suggested that one or two more intravenous injections were used than was necessary. Nevertheless, without the use of adjuvants, multiple injections are needed in rabbits. These results throw some light on the varied responses noted by other workers who used a small number of injections. It is possible that other doses or other schedules might give the same result with fewer injections, and these require investigation. Also, the use of adjuvants would probably give a reduction in the number of injections required, though avoidance of mineral oil adjuvants when developing a vaccine for human use is probably desirable.

The results of challenge experiments showed a number of interesting features:

1. The immunity produced by heated virus was sufficient to save rabbits from death or serious illness from rabbitpox.

2. The immunity to intradermal challenge with vaccinia or cowpox was intermediate between normal rabbits and those immunized with live virus.

3. On rechallenge there had been little or no increase in immunity following the initial challenge.

Challenge by intradermal inoculation of a quantity of live virus into an avascular region is a severe test of immunity. Humoral antibody will reach the site with

comparative difficulty, and may not be present in significant quantity until the infection is established with the development of inflammation and oedema. In a disease like smallpox, where the virus is disseminated by a viraemia, circulating antibody will play a large part in resistance. The failure of any of the rabbits immunized with heated virus to develop secondary lesions, viraemia or generalized illness is an indication that such immunity can be life saving. Judged only by the skin reactions to challenge, the immunity produced by heated virus could be said to be unsatisfactory, in the sense that Amies (1961) and RamanaRao (1962) found their rabbits to be poorly resistant. Had they used a different challenge virus they might have found their rabbits to be more immune than they thought.

That the lesions produced by challenge were not due to allergy was shown by the concomitant use of heated virus as a second 'challenge'. No lesions were seen, in contrast to McNeill's (1966) results. He noted delayed-type hypersensitivity with his heated virus controls. Both McNeill (1966) and RamanaRao (1962) noted necrosis at the sites of challenge of live virus, the latter being able to distinguish by this means those rabbits which had been immunized with killed virus from those receiving live virus. In the present study the heated virus appeared to *protect* from necrosis, rather than promote it, when compared with the response of unimmunized rabbits. These conflicting results are not explicable at present, but the interval between immunization and challenge may be important. Fulginiti, Arthur, Perlman & Kempe (1966) found severe local reactions to live measles when administered four years after killed virus, but not when live virus was given as part of the immunization schedule.

Failure of the animals immunized with heated virus to develop further immunity after challenge with live virus is interesting. If development of a lesion represents virus multiplication, then new antigens must have been formed locally. The failure to develop further detectable antibody and immunity to challenge suggests that recognition of such fresh antigen by the rabbits' defences is not a peripheral one, and that circulating antibody prevented its being registered by more central mechanisms. This would certainly have to be borne in mind when considering human application.

To summarize the present series of experiments, rabbits immunized with heated virus developed a detectable immunity which was sufficient to protect them from the more severe and lethal effects of challenge, but only following a course of multiple injections that would probably not be acceptable for use in man. It might be possible to combine the injections with other immunizations, but this would require careful assessment. It is both of interest and a caveat that the immunity developed may prevent a complete response to challenge (i.e. vaccination), and no information has been obtained as to the duration of immunity. It is worth noting that, following three doses of inactivated measles vaccine, the disease can still be caught and may even be more severe (Rauh & Schmidt, 1965).

Much further animal work remains to be done before even a pilot study could be carried out in man, but enough information has been obtained to suggest that it might be possible to develop a heated vaccine without the disadvantages and hazards of virus multiplication.

SUMMARY

Rabbits were immunized by multiple intradermal injections followed by six intravenous doses of vaccinia virus inactivated by heating to 65° C. Particular attention was paid to confirming that the virus used was fully inactivated and incapable of reactivation. The immunized rabbits developed neutralizing and other antibodies to a titre comparable with those developed in response to live virus, but multiple intravenous injections were required to elicit a maximum titre. A qualitatively different response was seen only in immunodiffusion tests in agar gel where two or three fewer lines developed with antisera to heated virus than with those to live virus. The rabbits were subsequently challenged intradermally either with vaccinia and cowpox or with rabbitpox. They showed some immunity to vaccinia and cowpox, compared with normal controls, but less than that elicited by live virus. Their resistance to lethal doses of rabbitpox was life-saving though some rabbits did develop lesions. Later rechallenge of the rabbits showed that they had not developed further immunity, in distinction from the normal controls. The implications of these findings are discussed.

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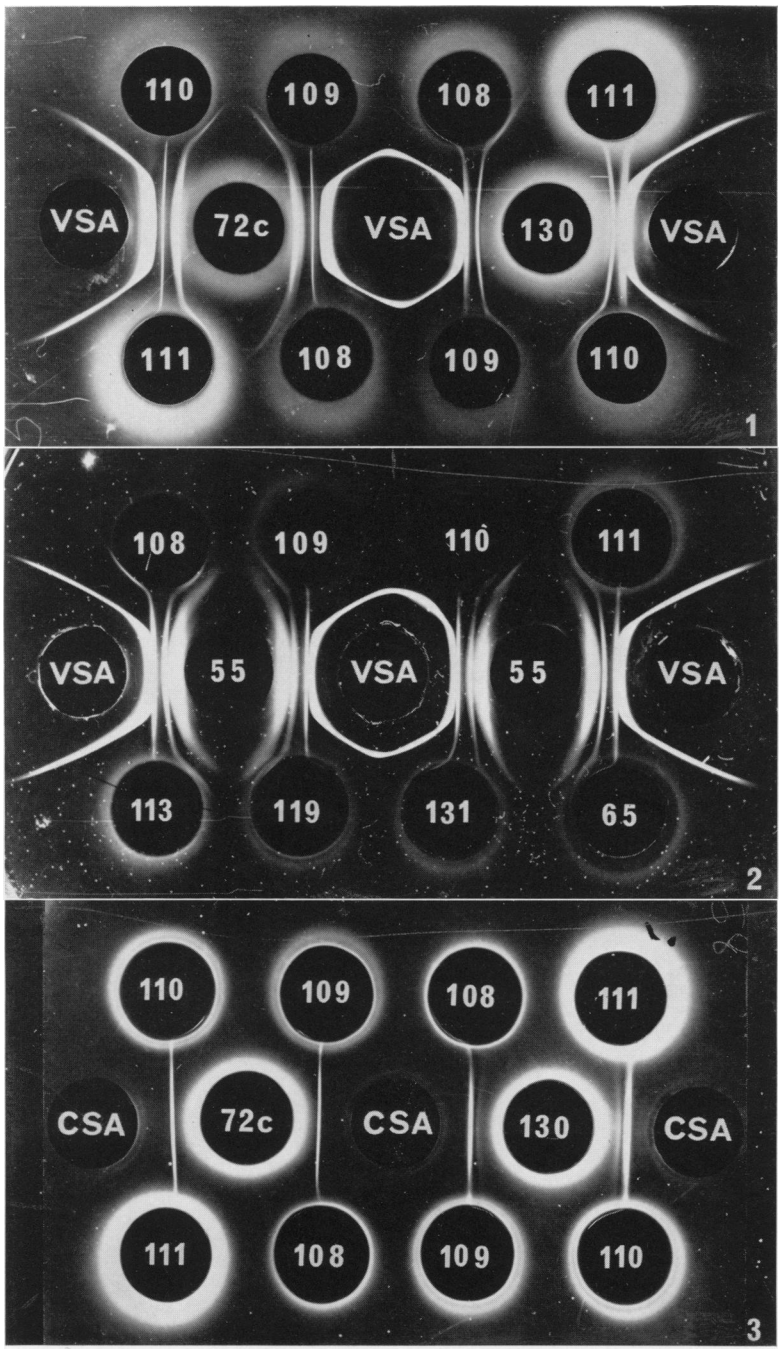
EXPLANATION OF PLATE

PLATE 1

Fig. 1. Line patterns given by antisera made against heated and live vaccinia virus. VSA, vaccinia soluble antigen. 72c and 130, sera made against live virus. 108–111, sera made against heated virus. The sera made against live virus give 1 or 2 more lines than those made against heated virus.

Fig. 2. Similar to Fig. 1. Serum 55 was made against live virus, and shows 2 or 3 more lines than those made against heated virus.


110
109
108
111
VSA
72c
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130
VSA
111
108
109
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72c
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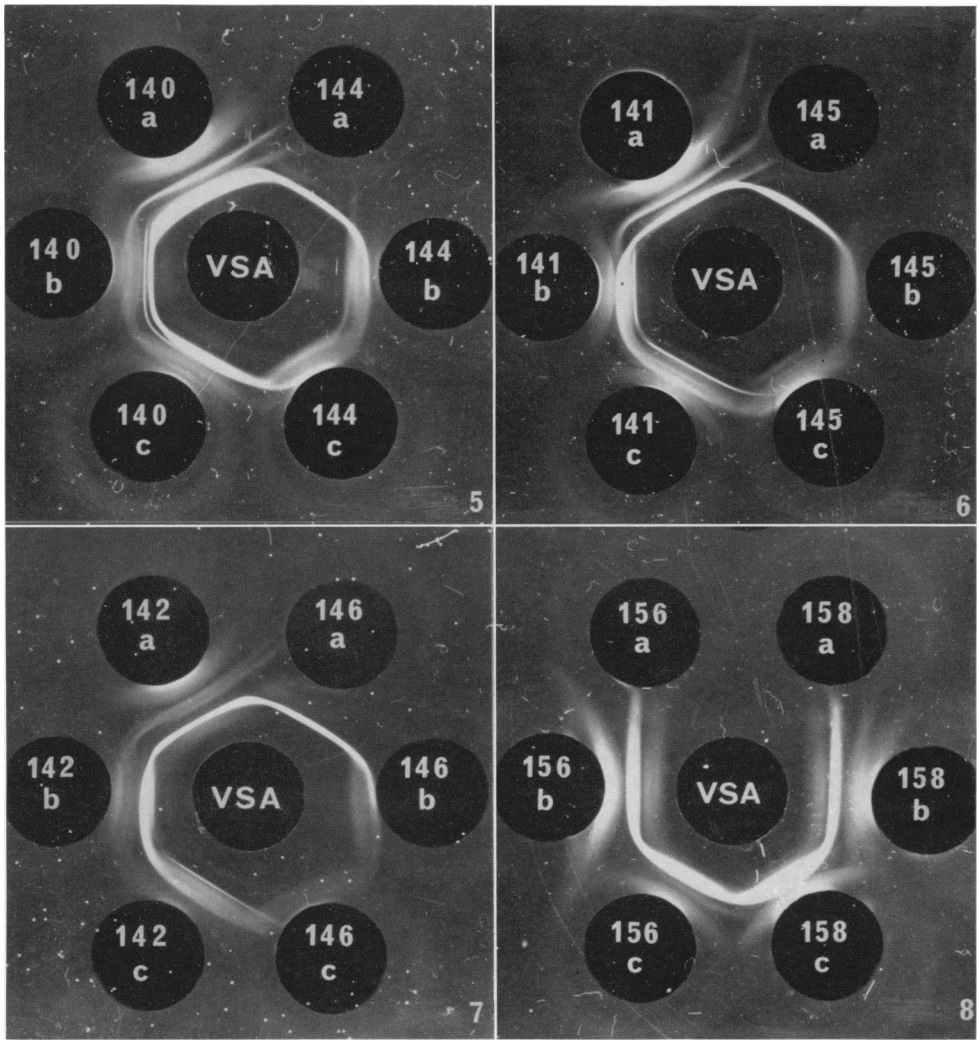
140 a
144 a
140 b
VSA
144 b
140 c
144 c
5
141 a
145 a
141 b
VSA
145 b
141 c
145 c
6
142 a
146 a
142 b
VSA
146 b
142 c
146 c
7
156 a
158 a
156 b
VSA
158 b
156 c
158 c
8

Fig. 3. Line patterns given by antisera to heated and live virus with cowpox antigen. CSA, cowpox soluble antigen. Sera as in Fig. 1. The sera made against live virus show one or two lines of precipitate but no lines are given by the antiheated virus sera. This point is discussed further in the text.

PLATE 2

Figs. 5–8. Comparison of pre- and post-challenge antisera. The *a*, *b* and *c* sera are those taken following immunization, after first challenge and after rechallenge. Rabbits 140 and 141 were immunized with live virus, and challenged twice. Rabbits 144 and 145 were immunized with heated virus and also challenged twice. Rabbits 142 and 146 were immunized with live and heated virus respectively and were not challenged. Rabbits 156 and 158 were normal unimmunized rabbits which were challenged twice.

VSA, vaccinia soluble antigen.

Figs. 5 and 6 show that the rabbits immunized with live virus (140 and 141) did not develop fresh lines following challenge and their line systems were multiple. The rabbits immunized with heated virus developed a single line before challenge with vaccinia and cowpox and added a second faint line after challenge. This is discussed in the text. Fig. 8, the control rabbits (156 and 158) developed multiple line patterns after initial challenge.