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The antibody response of rabbits to inactivated vaccinia virus

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INTRODUCTION

The often unpleasant and occasionally serious side effects of Jennerian vaccination have stimulated many studies on the antigenicity of inactivated vaccinia virus, with the object of using inactive preparations either as a substitute for live virus, or to provide a basic immunity which might protect against the illness of subsequent vaccination with live virus without interfering with its effectiveness.

Most investigations have been directed to the problem of immunogenicity of inactivated vaccinia virus, but the situation has not been clarified. For example, Parker & Rivers (1936) demonstrated some immunogenicity of formalin-inactivated vaccine in rabbits, but considered that this was weak and unlikely to be useful for the protection of humans against smallpox. More recently, Amies (1961) demonstrated what he considered to be a negligible immune response in rabbits given formalinized vaccine with adjuvant. In contrast RamanaRao (1962) reported that formalin-inactivated vaccine with adjuvant stimulated levels of virus neutralizing antibody in rabbits approaching those resulting from dermal infection with live virus. Previously Collier, McClean & Vallet (1955) had shown that an ultraviolet (UV) inactivated vaccine without adjuvant stimulated a regular immune response in rabbits. A similar vaccine (five times concentrated) tested in man was immunogenic in only about 50 % of subjects (Kaplan, McClean & Vallet, 1962). Kaplan (1962) extended the study of this UV-inactivated vaccine in humans and showed that 50 times the rabbit dose stimulated substantial increase in the level of virus neutralizing antibody in individuals who had previously been vaccinated with live vaccine.

There is no doubt therefore that inactivated vaccinia virus can be immunogenic. Precise evaluation of the conditions necessary to ensure immunogenicity is difficult, and this is largely due to the variety of methods and criteria of immunogenicity which have been used by different workers.

Several conditions could be of critical importance. The dose of virus antigen would seem to be most obvious, and this was stressed in some of the earlier work (Bernkopf & Kligler, 1937). However, with vaccines of apparently similar virus content, e.g. that reported by Collier *et al.* (1955) and that of Amies (1961), the former was satisfactory while the latter possessed poor immunogenicity. It is obvious that the inactivating agent could be of great importance, but again the effect is not clear since Kaplan (1960) has shown that even with the same dose of virus and the same inactivating agent (gamma radiation) one vaccine can be immunogenic, while another with a longer period of inactivation is not. The

duration of contact between virus and inactivant could therefore be extremely critical, whilst other conditions, e.g. the route of immunization, may obviously play an important part.

The work reported in this paper was an attempt to clarify the effect of these factors on the immunogenicity of inactivated vaccinia virus, measured by the neutralizing antibody response in rabbits, by studying them under the same experimental conditions, so that a comparison of their importance could be made.

Four series of experiments were performed with inactivated vaccinia vaccines:

(I) Vaccines with constant virus content but with differing methods and extent of inactivation.

(II) Vaccines with differing virus content and constant method and extent of inactivation.

(III) Vaccines as in series (II) but with virus suspended in polyvinylpyrrolidone (PVP) to assess the adjuvant effect of this substance.

(IV) Vaccines administered by different routes.

MATERIALS AND METHODS

Virus. The Lister Institute strain of rabbit-adapted vaccinia virus was used. It was propagated on the skin of rabbits by the method of Hoagland, Smadel & Rivers (1940).

Rabbits. Young adult New Zealand Whites, of both sexes, weighing 2–3 kg.

Infectivity titrations

Virus was titrated by plaque counting in monolayers of HEp_2 cells. Cell cultures were made in 12 oz. flat medicine bottles at 37° C. The growth medium was Eagle's basic medium modified to contain twice the concentration of amino acids and vitamins, and containing 10% tryptose phosphate broth (Difco) and 10% calf serum. The confluent monolayer of cells in a bottle was suspended in 75 ml. growth medium after 2 min. treatment with 5 ml. of a solution of 0·25% trypsin and 0·02% 'versene' at 37° C. One-ounce bottles were used for plaque counts; they were inoculated with 5 ml. of this cell suspension which after 28 hours at 37° C. had formed an almost complete monolayer; they were then ready for virus inoculation.

Virus dilutions were made in McIlvaine's buffer of 0·004 M, pH 7·2. Growth medium was poured off and the plaque bottles inoculated with 0·5 ml. of virus. Serum-free medium was added to bring the total volume of fluid in the bottle to 2·5 ml. The bottles were placed at 37° C. overnight for virus adsorption. The fluid was then poured off and the monolayers fed with 5 ml. fluid overlay medium (modified Eagle's medium containing 10% tryptose phosphate broth, 1% calf serum, antivaccinia serum, and additional bicarbonate buffer). The bottles were replaced at 37° C. for a further 2 days to allow plaque development. The medium was discarded and the monolayers stained with Ziehl-Neelsen's carbol fuchsin diluted 1/20. Two or three bottles were used for each dilution of virus.

Fluid overlay containing antiserum was used instead of a solid overlay to

prevent formation of secondary plaques. Antiserum can be used for this purpose since it is known that vaccinia virus spreads directly from cell to cell in a monolayer (Nishmi & Keller, 1962). This method has two important limitations: (i) virus released into the fluid medium may not be completely neutralized even when the antibody is present in excess, and (ii) infected cells may become detached and initiate secondary plaques by direct cell-to-cell spread. The antiserum overlay was therefore used only to gain an additional 24–48 hr. in plaque development time. The antiserum used was antivaccinia serum prepared in rabbits 3 weeks after they had recovered from a vaccinia infection over a wide area of skin by giving them three intravenous inoculations, at weekly intervals, of a clarified extract of rabbit-skin virus. The animals were bled out 1 week after the final inoculation and the sera pooled, sterilized by Seitz-filtration, and stored at $-20°$ C. The antiserum was used in the overlay medium at a dilution which prevented the appearance of secondary plaques for at least 24 hr. after the time at which the plaques were normally counted.

Preparation of virus suspensions

Virus from infected rabbit skin was purified by one cycle of differential centrifugation, followed by centrifugation in a sucrose density gradient using the method of Zwartouw, Westwood & Appleyard (1962). Sucrose was removed from the virus suspension by dialysis against two changes each of 1000 vol. of McIlvaine's buffer 0·004 M, pH 7·2, for a total of 6 hr. at 4° C.

Preparation of vaccines

(*a*) *Constant virus content, variable inactivant and extent of inactivation.* Sixteen vaccines were prepared from a pool of active virus, of which 15 fell into three groups according to the method of inactivation—hydroxylamine, formalin or heat. *Hydroxylamine* is thought to react exclusively with nucleic acid (Lie, 1964). It was used in these experiments as a convenient method of inactivating virus with minimal interference with protein antigens. A solution of 2×10^{-3} molar was made up immediately before use. This was mixed with an equal volume of purified virus and inactivation took place in the dark at room temperature. *Formalin* in low concentrations was used to inactivate virus by a relatively mild effect upon protein antigens. The method used was that of Gard (1957). Purified virus was mixed with an equal volume of 0·12 M formalin, 0·04 M glycine and allowed to react in the dark at room temperature. *Heat* inactivation was used to cause more damage to viral protein antigens than the others and was achieved by placing tubes containing purified virus in a water bath at 50° C. Preliminary inactivation curves were obtained in order to determine suitable inactivation times for the production of vaccines. For each method of inactivation five vaccines were prepared at stages during the course of inactivation. Virus was removed from the inactivant by centrifugation in a Spinco L ultracentrifuge at 30,000 rev./min. for 30 min. The virus 'pellet' was resuspended by ultrasonic vibration in McIlvaine's buffer 0·004 M, pH 7·2, containing 10 % polyvinylpyrrolidone.* This substance was used

* 'Plasdone C'; supplied by Fine Dyestuffs and Chemicals Ltd., Manchester.

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because of its stablizing effect on virus infectivity and adjuvant effect (Amies, 1962). In order to preserve uniformity the heat inactivated virus was treated exactly as the hydroxylamine and formalin inactivated virus. Table 1 shows the residual virus infectivity (pfu/ml.) of each of these vaccines.

Table 1. *Method and extent of inactivation for each vaccine in the first series*

	Vaccine	Temp.	Titre (pfu/ml.)
1	Active	—	2×10^7
2	Formalin 12 hr.	19° C.	6×10^3
3	Formalin 36 hr.	19° C.	3×10^1
4	Formalin 48 hr.	19° C.	Nil
5	Formalin 96 hr.	19° C.	Nil
6	Formalin 192 hr.	19° C.	Nil
7	Hydroxylamine 3 hr.	19	$1{\cdot}6 \times 10^3$
8	Hydroxylamine 5 hr.	19	10
9	Hydroxylamine 7 hr.	19	2
10	Hydroxylamine 16 hr.	19	Nil
11	Hydroxylamine 48 hr.	19	Nil
12	Heat 30 min.	50	$6{\cdot}4 \times 10^2$
13	Heat 2 hr.	50	10
14	Heat 4 hr.	50	2
15	Heat 7 hr.	50	Nil
16	Heat 24 hr.	50	Nil

Table 2. *Estimated virus content of inactivated vaccines based on infectivity before inactivation*

Vaccine	Virus content pfu/dose (0·5 ml.)
17	$2{\cdot}8 \times 10^8$
18	7×10^7
19	$1{\cdot}8 \times 10^7$
20	4×10^6

(*b*) *Variable virus content vaccines in buffer.* Four formalin inactivated vaccines were prepared by mixing equal volumes of purified virus with 0·012 M formalin, 0·04 M glycine, and allowing inactivation to take place in the dark at room temperature for 100 hours. Virus was removed from the formalin by centrifugation at 30,000 rev./min. for 30 min. in the ultracentrifuge, and the virus 'pellet' resuspended in McIlvaine's buffer 0·004 M, pH 7·2 by ultrasonic vibration. Allowing for losses during centrifugation the approximate virus content of each vaccine was as shown in Table 2.

Each vaccine was tested for residual infectivity by inoculation onto the chorioallantoic membranes of 12-day-old chick embryos, using 4 eggs per vaccine and 0·1 ml. of inoculum. Active virus was not detected in any of the vaccines.

(*c*) *Variable virus content vaccines in PVP.* Three similarly prepared formalin-inactivated vaccines were suspended in McIlvaine's buffer pH 7·2 containing

10 % PVP. The approximate virus content of each of these vaccines is shown in Table 3.

Infectivity testing of these vaccines on the chorioallantoic membrane did not reveal any active virus. All vaccines were dispensed in 0·5 ml. amounts in sealed ampoules and stored at −70° C.

Table 3. *Estimated virus content of inactivated vaccines containing polyvinylpyrrolidone, based on infectivity before inactivation*

Vaccine	Virus content pfu/dose (0·5 ml.)
21	9×10^7
22	3×10^7
23	6×10^6

Immunogenicity testing

The immunogenicity of the vaccines was assessed by measurement of virus neutralizing antibody produced by rabbits following two intramuscular inoculations of 0·5 ml. of vaccine. In series (I) (variable inactivation) there was an interval of 4 weeks between inoculations: blood was obtained before and 2 weeks after each inoculation. In the second, third and fourth series the inoculations were given 6 weeks apart and the animals bled before inoculation, and at 5, 10, 20, 30 and 42 days after this. They were bled again 14 days after the second inoculation. Each individual vaccine was tested in four rabbits.

Measurement of neutralizing antibody

Appropriate twofold dilutions of serum were made in dilute McIlvaine's buffer 0·004 M, pH 7·2, in the unit volume of 0·3 ml. An equal volume of test virus was added and the mixture incubated for 2 hr. at 37° C. Monolayers of HEp_2 cells in 1 oz. plaque bottles were inoculated with 0·2 ml. virus–serum mixture using two bottles for each serum dilution. After absorption of unneutralized virus the monolayers were overlaid and incubated as described for plaque assay. A virus control consisting of 1 vol. of test virus and one volume of buffer was included in each batch of tests. The control mixture was incubated for 2 hr. at 37° C. and 0·2 ml. volumes inoculated into each of four plaque bottles. The average plaque count in the control bottles was taken as 100 % virus survival and the titre of a serum was expressed as the reciprocal of the dilution of serum which allowed 50 % virus survival. The test virus suspension was vaccinia virus grown in HEp_2 cells and partially purified by differential centrifugation. This virus was suspended in 20 % skim milk in dilute McIlvaine's buffer and stored in 1 ml. amounts at −70° C. For each batch of tests one of these bottles was thawed and further diluted in 20 % skim milk in dilute McIlvaine's buffer to give a virus control plaque count of approximately 100. Skim milk was used in the suspending medium for virus in the neutralization test in order to prevent heat inactivation of the virus during incubation of virus serum mixtures (Boulter, 1957).

RESULTS*

First series of experiments

The most striking feature of these results was the great variation in the titres of antibody obtained even within a group of rabbits receiving the same vaccine. Figure 1 shows the levels of antibody for each rabbit 2 weeks after the first dose of vaccine, and also the residual virus infectivity (pfu/ml.) in each vaccine. Most rabbits had developed negligible quantities of antibody ($< 1/10$), but a few, especially those receiving hydroxylamine inactivated vaccines, developed antibody to a much higher titre.

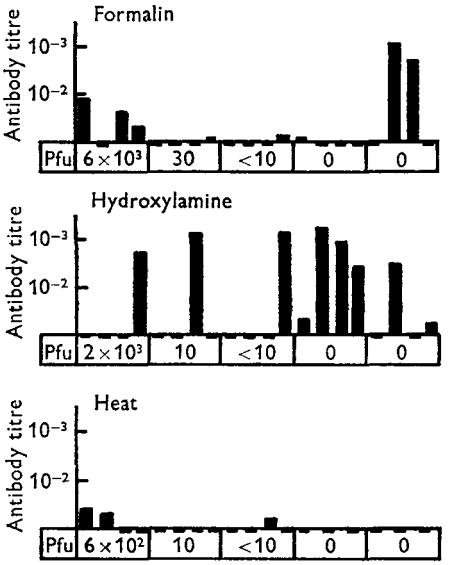

Fig. 1. Neutralizing antibody titres in rabbits 2 weeks after a single inoculation of formalin, hydroxylamine or heat inactivated vaccines. Residual virus infectivity of each vaccine is given in pfu/ml.

Second series of experiments

A possible explanation for the variable results obtained in the first series was that the vaccines contained antigen within the critical concentration range, resulting in a poor and transient antibody response in some rabbits and a good and more enduring response in others (Svehag & Mandel, 1964). This series was undertaken to determine whether or not this type of variable antibody response occurred with inactivated vaccinia virus, and within what range of virus concentrations. The antibody responses obtained were of two types: (i) a poor response characterized by a low titre maximal 5 days after inoculation which declined to undetectable levels by 10–20 days, and (ii) a good response characterized by a higher titre maximal 10 days after inoculation, antibody being detectable 6 weeks after inoculation.

* Complete tables of results are available in the author's M.D. thesis, The Queen's University of Belfast.

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Third series of experiments

The maximum antibody titres obtained after one inoculation in good responses in the second series (1/190) were much lower than the highest titres developed by some rabbits in the first series (1/2000). This difference might have been due to an adjuvant effect of the PVP contained in the vaccines of the first series. To clarify this and to evaluate the adjuvant property of PVP the third series of experiments was undertaken. The results again showed the two types of antibody response, but neither the incidence of good responses in relation to the concentration of virus in the vaccines nor the antibody titres obtained were any higher than those achieved with similar vaccines which did not contain PVP.

Fourth series of experiments

A comparison was made of the antibody response to vaccine 17 (Table 2) when given intradermally (I.D.) or subcutaneously (S.C.) and that following intramuscular (I.M.) inoculation. All inoculations (0·5 ml.) were made into the left thigh. The intradermal vaccine was divided into 5 inocula of 0·1 ml. and these were all

Table 4. *Geometric mean of neutralizing antibody titres 2 weeks after the second dose of vaccine 17 when given by different routes*

Route	Geometric mean titre
I.M.	887
S.C.	5175
I.D.	5549

given into as small an area of skin as was possible. The high dose vaccine was used in the expectation that all rabbits would show the good type of antibody response, and therefore make a more valid comparison of antibody titres between the groups. With the exception of one rabbit in the S.C. group all rabbits showed the good type of antibody response. The antibody titres 2 weeks after the second inoculation indicated that the most uniform response followed I.D. inoculation. Titres following I.M. inoculation were much lower, and those following S.C. inoculation were much more variable, although if the rabbit which showed the poor transient response is excluded, the geometric mean titre of antibody after two inoculations of vaccine was similar to that following I.D. inoculation. Geometric mean titres for each group are shown in Table 4.

Summary of antibody responses in relation to dose of antigen

The small number of rabbits which received each individual vaccine makes it impossible to evaluate each vaccine precisely. The combination of data from the second, third and fourth series of experiments clearly shows the importance of the quantity of antigen in determining the immunogenicity of these vaccines. Table 5 shows the relationship between the antigen content of each vaccine, based on pfu of infectivity before inactivation, and the incidence of good antibody responses in each group of vaccines. This also shows that variable antibody responses are associated with the virus concentration range 10^7–10^8.

Table 5. *Relationship between concentration of virus (pfu/0·5 ml. before inactivation) and incidence of good antibody responses*

Virus content of vaccine (pfu/0·5 ml.)	Incidence of good antibody responses
$2{\cdot}8 \times 10^8$	11/12
9×10^7	2/3
7×10^7	3/4
3×10^7	2/4
$1{\cdot}8 \times 10^7$	1/4
6×10^6	0/4
4×10^6	0/4

DISCUSSION

Rather than helping to clarify the position of the inactivating agent and the extent of inactivation in the immunogenicity of inactivated vaccinia virus, the results of the first series of experiments seem to add further confusion to the problem. Most of the rabbits developed low titres of antibody, but a few developed a much higher titre, especially in the hydroxylamine-inactivated vaccine series. The occurrence of these high reactors did not seem to bear any relationship to the degree of inactivation, and even within a group of rabbits receiving the same vaccine some developed high titres of antibody and others very low titres. The most obvious explanation is that the higher titres resulted from infection with live virus either by incomplete inactivation, by some reactivation process, or by accidental infection. But these possibilities seem unlikely since the last two vaccines in each series were devoid of infectivity for either the chorioallantoic membrane or for tissue culture, and even when a partially inactivated vaccine with a residual infectivity of approximately 10^3 pfu was inoculated, not all rabbits showed the high antibody response. Accidental dermal infection at the site of inoculation was not seen in any of the rabbits, and in order to minimize the risk of accidental infection by other means the rabbits which received totally inactivated vaccines were kept in a separate building. Furthermore none of the preimmunization sera from these rabbits showed detectable antibody activity. If these results are not easily explicable in terms of infection with live virus, is there any alternative explanation?

Svehag & Mandel (1964), using poliovirus as antigen, have shown that in rabbits there can be variable antibody responses to the same quantity of antigen, provided the quantity of antigen is within a certain range. Below this range all rabbits showed a low and transitory response and above it all rabbits showed a higher and more enduring response. Within the critical range some rabbits gave the low and others the high response, the type of response being dependent upon the individual rabbit. Assuming that the quantity of antigen was within this critical range a similar phenomenon with inactivated vaccinia virus could explain the results of the first series of experiments, and also explain the variability in

effectiveness of inactivated vaccinia virus vaccines which has been such a feature of the literature.

The results of the second and third series of experiments show (i) that this variable response occurs with inactivated vaccinia virus vaccines when the quantity of antigen corresponds to an infectivity titre of approximately 10^7 to 10^8 pfu before inactivation, but (ii) that the titres obtained in good antibody responses were still lower than those high titres obtained in the first series, and (iii) that this was not due to any adjuvant effect of the PVP in the first series of vaccines.

It is difficult to avoid the conclusion that the animals showing the high titres of antibody in the first series had become accidentally infected with vaccinia virus at some time between the taking of the preimmunization serum and the first dose of vaccine although for the stated reasons it is difficult to see how this could have happened. That accidental infection of rabbits with vaccinia virus can easily occur, in spite of precautions taken to prevent it, has been pointed out by Parker & Rivers (1936). Whatever the explanation for these high titres may be, it is clear that a single antibody measurement taken at an arbitrarily chosen time within 2–3 weeks after administration of antigen can involve difficulties in interpretation. If the dose of antigen is within the critical range where variable antibody responses may occur, and this dose is varied unintentionally, even within small limits, the calculation of mean antibody titres in relation to some other factor may lead to erroneous conclusions. The experiments on the route of immunization indicate that the highest and most uniform antibody response was obtained by intradermal inoculation, assuming that the division of the intradermal doses was unimportant. Antibody responses following subcutaneous inoculation were very variable and one animal in this group showed a poor response to the first dose of vaccine. Whether this was an immunologically refractory rabbit or whether it indicated a lesser efficiency of the subcutaneous route is a problem which would obviously require to be studied in a much larger number of animals.

In conclusion, the dose of virus is the most important single factor in determining the immunogenicity of inactivated vaccinia virus vaccines when the immunogenicity is assessed by the development of virus neutralizing antibody. If the high antibody titres found in the first series of experiments can be discounted on the ground that the rabbits may have been accidentally infected with live virus, it can be concluded that neither the method nor the degree of inactivation within the limits tested plays a major part in determining immunogenicity. The intradermal route of immunization would seem to have the advantage of providing both a uniform and a high level of antibody production compared with either the intramuscular or subcutaneous routes.

If an inactivated vaccinia virus vaccine of assured immunogenicity can be produced there are many problems in its practical application. It has been shown by Beunders, Driessen & van den Hoek (1960) that primary immunization with inactivated vaccine confers considerable protection against the illness of subsequent Jennerian vaccination. Herrlich (1964) has reported that primary immunization with inactivated vaccine results in a lower incidence of post-vaccinal

encephalitis following subsequent vaccination, though lack of control groups in this study must limit its value.

Even if it can be shown conclusively that primary immunization with inactivated vaccine can protect against most of the complications of live virus vaccination, it would be of great importance to determine the immunization schedule which would strike a balance between maximal protection from the vaccination illness, and minimal interference with the effectiveness of vaccination. It would be undesirable to induce a degree of skin immunity with the inactivated vaccine which would make it more difficult to get a subsequent vaccination 'take'. Bearing this in mind it would be interesting to discover whether or not a rapid though transient antibody response to inactivated vaccine would be sufficient to protect against the 'illness of vaccination' if live and inactivated vaccine were given simultaneously. It is probable, however, that many of those who develop vaccinia gangrenosa or generalized vaccinia have immunological defects, and it is unlikely that primary immunization with inactivated vaccine will benefit such people.

The major problem in using an inactivated vaccine as a substitute for Jennerian vaccination is to show that it can be effective. The one indubitable fact about Jennerian vaccination is that it protects against smallpox. The mechanism of this protection is, however, poorly understood. No generally acceptable laboratory criteria are therefore available by which the results of any major change in the method of vaccination can be assessed, be it the use of a further-attenuated live virus, an inactivated vaccine, primary immunization with inactivated vaccine or the simultaneous use of gamma globulin or an anti-viral drug. If sound laboratory criteria for the assessment of immunity to smallpox were available, this, in conjunction with the epidemiological data available, would provide a firm basis for the formation of scientifically acceptable vaccination policies.

SUMMARY

Inactivated vaccinia virus vaccines were prepared from purified virus inactivated by either formalin, hydroxylamine or heat. The immunogenicity of these vaccines was assessed in rabbits by measurement of virus neutralizing antibody following each of two inoculations. It was concluded that inactivated vaccinia virus stimulates the production of neutralizing antibody and that the most important single factor in this immunogenicity is the concentration of virus in the vaccine. Vaccines prepared from virus suspensions containing 10^7 to 10^8 pfu/ml. before inactivation give variable antibody responses.

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