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The development of skin resistance and hypersensitivity following inactivated vaccinia virus vaccines in rabbits

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INTRODUCTION

Skin resistance or skin immunity to vaccinia virus is a well recognized phenomenon, although its mechanism is not understood. It is a matter of common observation that it can be more difficult to produce a vaccination 'take' in previously vaccinated than in non-vaccinated individuals. Though development of skin immunity after infection with live vaccinia virus is well established, there is considerable difference of opinion concerning the development of skin immunity as a result of immunization with inactivated vaccinia virus.

According to some investigators (Andrewes, Elford & Niven, 1948; Amies, 1961; RamanaRao, 1962) inactivated vaccinia virus vaccines do not produce an increase in skin immunity. There are others who consider that inactivated vaccinia virus can induce skin immunity but that there is no apparent relationship between this and the level of circulating antibody, (Kaplan, 1960; Beunders, Driessen & van den Hoek, 1960). Thirdly, it is believed that inactivated vaccinia virus can produce both circulating antibody and skin immunity (Parker & Rivers, 1936; Collier, McClean & Vallet, 1955). In order to investigate the development of skin resistance following immunization with inactivated vaccinia virus, and the relationship of this skin resistance to circulating antibody, it is necessary in the first place to define skin immunity, and on this basis to use an appropriate method for measuring it. It seems likely that different concepts of the nature of skin immunity and therefore the utilization of different methods for measuring it have contributed to the confused picture outlined above. Skin immunity can be defined as the resistance of the skin to the initiation of a focus of multiplication by a given inoculum of virus. Thus for each experimental animal one can determine, by a virus titration in the skin, the minimal inoculum of virus which will initiate a focus of multiplication. With this quantitative method difficulties of interpretation arise in deciding whether a skin reaction to virus represents a focus of multiplication or whether it represents a hypersensitivity reaction to the inoculated virus without multiplication necessarily having taken place. This difficulty can be overcome by using a control series of inactivated virus inocula in each animal, the quantity of virus in each inactivated dilution being identical with that in the corresponding live dilution. A larger reaction of longer duration at the site of challenge with live virus would indicate that multiplication had taken place. This method was used in the present study and is described in detail under Methods.

An alternative qualitative method for measuring skin immunity has been

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frequently used which involves challenging the skin with a single dose of high-titre virus and recording the results as a primary, accelerated, or immune response, or by some similar notation. By this method conversion from primary type reaction to an accelerated reaction may be interpreted as an index of skin immunity. Such an interpretation could possibly imply that delayed-type hypersensitivity is an important factor in skin immunity. This would be based on the possibility that a primary reaction and an accelerated reaction are similar foci of viral multiplication, the accelerated reaction being accelerated because the subject possessed residual delayed hypersensitivity. This raises another question, namely, is there any relationship between the degree of skin resistance and the degree of delayed-type hypersensitivity? The present investigation was undertaken in an attempt to clarify these problems. Two questions were asked:

(i) Is there any increase in skin immunity following immunization with inactivated vaccinia virus?

(ii) If skin immunity does develop, is this related to either the level of circulating antibody or the degree of delayed hypersensitivity?

METHODS

Skin tests were performed in the rabbits which had been used for the study of antibody responses to inactivated vaccinia virus (McNeill, 1965). Methods for preparation of the vaccines, titration of infectivity, titration of virus neutralizing antibody, and immunization of rabbits were as previously described. Skin testing was performed in each rabbit 2 weeks after a second dose of inactivated vaccine. Sera were collected before inoculation of skin-testing virus and titrated for neutralizing antibody.

Skin testing

Rabbits were tested by intradermal inoculation of dilutions of active and inactivated virus in the shaved skin of their backs. Each inoculum was 0·1 ml. Preliminary experiments were performed in order to determine the end-point range with serial tenfold dilutions of live virus in normal and previously vaccinated rabbits. From these experiments it was concluded that a suitable range of titres for test virus was 10^4—10 plaque forming units (pfu) per inoculum.

Preparation of skin-testing virus

Vaccinia virus grown on Hep 2 cells was partially purified by one cycle of differential centrifugation. Virus was diluted to 10^5 pfu/ml. and divided into two equal parts, one of which was inactivated by heat at 60° C. for 1 hr. Two tenfold, followed by two fourfold dilutions were made of both the active and inactivated suspensions. The fourfold dilutions were made in order to give a more precise end-point in the skin titrations. Dilutions of active virus were dispensed in 1 ml. amounts and stored at −70° C., and dilutions of inactive virus were dispensed in 20 ml. amounts and stored at 4° C. Each rabbit was inoculated with each dilution of active and inactive virus, active virus being inoculated on the right side of the back, and inactive virus on the left side. The inactive virus not only served as a control for

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the hypersensitivity reaction to the quantity of virus inoculated, but it also allowed an estimation of the degree of hypersensitivity by determining the highest dilution of inactivated virus which gave rise to a reaction. The diameter of induration following each inoculation was recorded daily for 5 days. A reaction to active virus which persisted at least 24 hr. longer than the reaction to the identical quantity of inactive virus, and which gave an indurated lesion greater by 2–3 mm. than the lesion from the inactive virus, was interpreted as evidence that some viral multiplication had taken place.

RESULTS

Table 1 gives the complete results for a normal rabbit and an immunized rabbit to illustrate the interpretation of the skin tests and the measurement of skin resistance and hypersensitivity.

Table 1. *The diameter of induration in mm. following intradermal inoculation of dilutions of active* (A_1–A_5) *and inactive* (I_1–I_5) *virus in a normal rabbit and in a rabbit previously immunized with inactivated vaccine*

Normal rabbit

Titre...	10^4 pfu/dose		10^3 pfu/dose		10^2 pfu/dose		25 pfu/dose		6 pfu/dose	
Day	A_1	I_1	A_2	I_2	A_3	I_3	A_4	I_4	A_5	I_5
1	7	—	4	—	—	—	—	—	—	—
2	9	—	7	—	5	—	3	—	2	—
3	12	—	9	—	8	—	5	—	3	—
4	15	—	11	—	10	—	7	—	4	—
5	9	—	6	—	7	—	7	—	5	—

Immunized rabbit

Titre...	10^4 pfu/dose		10^3 pfu/dose		10^2 pfu/dose		25 pfu/dose		6 pfu/dose	
Day	A_1	I_1	A_2	I_2	A_3	I_3	A_4	I_4	A_5	I_5
1	8	4	3	4	2	2	—	—	—	—
2	11	2	5	2	4	—	—	—	—	—
3	15	—	7	—	2	—	—	—	—	—
4	15	—	7	—	2	—	—	—	—	—
5	10	—	6	—	2	—	—	—	—	—

It can be seen that the normal rabbit reacted to every dilution of active virus, but not to any of the dilutions of inactive virus. It is therefore recorded as having a skin resistance of 0, and a degree of hypersensitivity of 0. Six normal rabbits were tested, and all reacted to the highest dilution of live test virus. The immunized rabbit, on the other hand, showed both skin resistance and hypersensitivity. The A_3 lesion in this rabbit was the lowest dilution to give a reaction, and this was clearly greater than the I_3 lesion. Taking the lowest dose (A_5) as unity, according to the previously stated dilutions this represents 16 times the amount of virus required to produce a lesion in normal rabbits. The rabbit was therefore recorded

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as having a skin resistance of 16. To record the degree of hypersensitivity, a reaction to the most concentrated suspension only (I_1) was recorded as 1 and to the other dilutions as 10, 100, 400 and 1600 respectively. This rabbit, which reacted to I_3, was recorded as having a degree of hypersensitivity of 100. These figures are obviously only relative, but nevertheless serve a useful purpose in that they provide at least a semi-quantitative estimation of two factors which are difficult to measure accurately.

Using this method the degree of skin resistance was determined for ninety-seven rabbits which had received two doses of various inactivated vaccinia virus vaccines. These experiments showed quite clearly that skin resistance to vaccinia virus can be increased by the administration of inactivated vaccine, although in many animals this increase was minimal, requiring only a fourfold increase in the amount of challenge virus to overcome it. The number of different vaccines used, and the small number of rabbits which received each vaccine, makes a calculation of the percentage of rabbits which developed skin immunity quite meaningless. However, as it has been shown that the most important factor in the immunogenicity of inactivated vaccinia virus in terms of development of neutralizing antibody was the dose of antigen in the vaccine (McNeill, 1965) the question arises as to whether development of skin resistance is also related to the dose of antigen. Table 2 shows the relevant data from thirty-five rabbits. No distinction is made in this table between degrees of skin resistance.

Table 2. *Relationship between virus concentration in vaccine (pfu/0·5 ml. before inactivation) and development of increased skin resistance*

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

It appears therefore that both skin resistance and the type of antibody response are dependent upon the dose of antigen used to immunize.

Is the degree of skin resistance related to the level of circulating antibody? Figure 1 shows the relationship in the form of a scatter diagram drawn from data obtained by tests on ninety-seven rabbits. This shows a definite although broad relationship between these factors. Figures 2 and 3 show that there is no relationship between the degree of hypersensitivity and either the degree of skin resistance or the level of circulating antibody.

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Unusual skin reactions

An unexpected feature of the skin testing was that some rabbits developed very severe skin reactions at the sites of inoculation of live virus. These severe reactions were characterized by the development within 36–48 hr. after inoculation of a

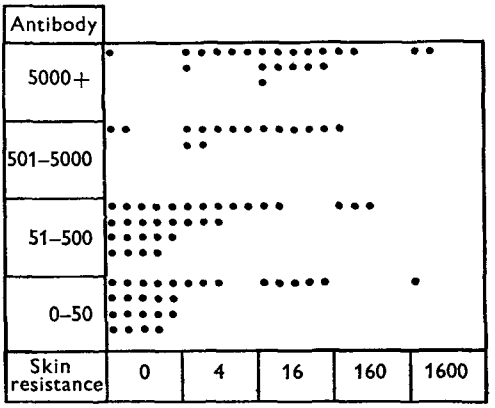

Fig. 1. A scatter diagram to show the relationship between virus neutralizing antibody and skin resistance to vaccinia virus following immunization with inactivated vaccinia virus vaccines.

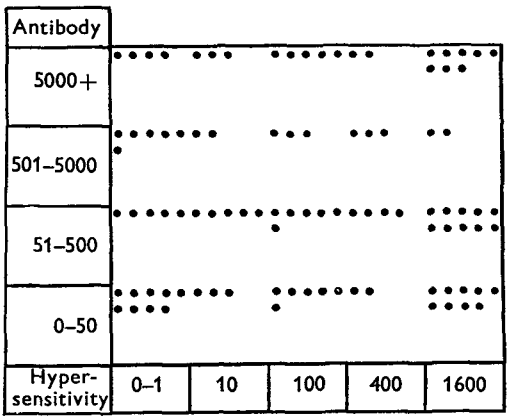

Fig. 2. A scatter diagram to show the relationship between virus neutralizing antibody and delayed hypersensitivity to vaccinia virus following immunization with inactivated vaccinia virus vaccines.

circumscribed indurated lesion with a surrounding area of intense erythema. By the third or fourth day the indurated area had become haemorrhagic and necrotic. Plate 1*a* shows one of these reactions on the sixth day, and Plate 1*b* a normal reaction to vaccinia virus at this stage for comparison. These severe reactions were not seen at any of the sites of challenge with inactivated virus. Histologically the predominant feature was an intense cellular infiltrate of the skin and subcutaneous tissue. This infiltrate was mainly composed of monocytes with a few eosinophils and plasma cells. Islands of perivascular lymphocytic infiltration were seen in some sections.

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The most severe reactions occurred in the series of experiments with vaccines prepared by using different inactivating agents and different degrees of inactivation (McNeill, 1965). There was an obvious difference in the severity of the reaction in that rabbits immunized with hydroxylamine-inactivated vaccines showed much more severe reactions than those immunized with either formalin or heat-inactivated vaccines. This is shown in Table 3.

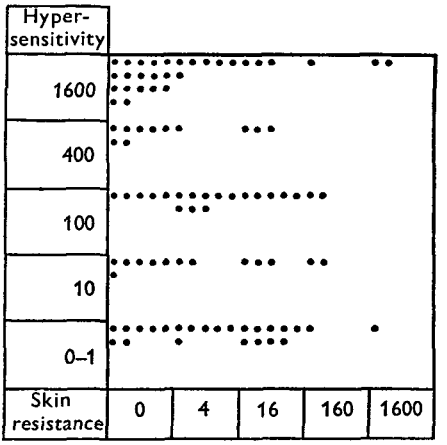

Fig. 3. A scatter diagram to show the relationship between skin resistance and delayed hypersensitivity to vaccinia virus following immunization with inactivated vaccinia virus vaccines.

Table 3. *Average diameter in mm. of the indurated lesion following I.D. inoculation of* 10^4 *pfu virus in rabbits showing the severe reaction*

(Measurements 5 days after inoculation.)

Formalin (nine severe reactors)	Hydroxylamine (seven severe reactors)	Heat (two severe reactors)
13	25	15

Table 4. *Incidence of severe reactions following skin challenge in rabbits immunized with inactivated vaccinia virus vaccine suspended in either buffer or polyvinylpyrrolidone*

Vaccine base	Severe reactions	Percentage
PVP	21/72	30
Buffer	2/25	8

The presence of polyvinylpyrrolidone (PVP) in the immunizing vaccine appeared to be a contributory factor. Table 4 shows the incidence of severe reactions in rabbits receiving PVP vaccines compared with those receiving vaccines suspended in buffer alone.

There was a tendency for rabbits with lower levels of circulating antibody to show this type of reaction, as shown in Table 5.

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Table 5. *Incidence of severe reactions in relation to the level of circulating antibody*

Neutralizing antibody titre	Severe reactions	Percentage
0–20	8/19	43
21–200	8/29	28
201–2000	6/24	25
2000+	1/25	4

DISCUSSION

The results of these experiments indicate that inactivated vaccinia virus vaccines can produce an increase in skin resistance to live virus in rabbits, and that the development of this skin resistance is dependent upon the quantity of antigen in the vaccine. Furthermore, the degree of skin resistance is related to the level of circulating neutralizing antibody and not to the degree of delayed hypersensitivity. These results are in apparent conflict with results reported by several other workers which were referred to in the introduction to this paper.

In considering these discrepancies there are three factors which should be considered. First, it has been shown in Table 2 that development of skin resistance is dependent upon the dose of antigen in the vaccine, and that regular production of skin resistance was produced only by vaccines containing approximately 10^8 pfu/dose infectivity before inactivation. Such concentrations of virus are greater than were used by many other workers. Secondly, it can be seen from Fig. 1 that the relationship between skin resistance and antibody titre is not a precise one, and is therefore likely to be apparent only when relatively large numbers of animals are studied. Experiments with sub-optimal doses of antigen in small numbers of animals could easily result in only a few animals developing skin resistance, and no apparent relationship between this and the titre of antibody. Thirdly, as previously explained, the method of assessing skin resistance could be important. It is interesting to note that several authors who found no relationship between skin resistance and titre of antibody had measured skin resistance by a qualitative method. The observations reported here show that there is no relationship between the degree of hypersensitivity and either the level of circulating antibody or the degree of skin resistance, and offer a possible explanation of why qualitative estimation of skin immunity in which hypersensitivity reactions are intimately involved may not show any relationship with levels of circulating antibody.

The severe florid reactions which were shown by some animals to challenge virus were quite unexpected. The rabbits which showed this type of reaction to live virus did not show any abnormality in their response to inactivated skin-testing virus. This may have been due to there being an insufficient quantity of antigen, since viral multiplication was necessary to provide an adequate stimulus for the development of this reaction. Of the rabbits showing severe reactions to live virus, 50 % reacted to all dilutions of inactive virus, compared with 30 % of normally reacting

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rabbits. The remaining 50% of severe reactors showed varying degrees of hypersensitivity to the inactivated test virus. This reaction was therefore more than a quantitative exaggeration of the normal reaction, it was also qualitatively different in that a pronounced 'vasculo-necrotic' element was present. The reactions were most severe in rabbits previously immunized with hydroxylamine-inactivated vaccines; the method of inactivation which theoretically caused least damage to the immunizing viral antigens. They were associated with the presence of PVP in the vaccines, this being the only manifestation of any adjuvant activity by this substance which was found (McNeill, 1965). The rabbits which developed the reaction tended to have lower levels of circulating antibody. Whatever the precise classification or aetiology of this severe reaction may be, it could be of practical importance particularly from the point of view of using inactivated vaccines before live vaccine. It is clearly possible that under certain circumstances, reactions to live vaccine after immunization with inactivated vaccine could be worse than primary vaccination with live vaccine alone.

Several reports of unusual skin reactions to vaccinia virus after immunization with inactive vaccine have appeared in the literature, e.g. Bussel & Mayzner (1930) reported two children who developed markedly erythematous and indurated lesions after Jennerian vaccination following three inoculations of formalin-inactivated virus. The lesions were much more severe than those resulting from the use of the same vaccine in previously unimmunized children. Weil & Gall (1940) noted that two rabbits showed a much more necrotic reaction to challenge virus after immunization with inactivated vaccine than was usual. It is interesting to note that in contrast to the other rabbits in the experiment one of these severe reactors had no detectable circulating antibody, and the other had a very low titre. Ehrengut (1959) described a peculiarity of the vaccination reaction in 4% of children who had previously been immunized with inactivated vaccine. This 'Hügelreaktion' was interpreted as an abnormal hypersensitivity reaction to the virus. RamanaRao (1962) was unable to demonstrate any quantitative increase in skin resistance following either live or inactivated vaccines, but intradermal challenge with live virus differentiated rabbits which had been immunized with inactivated vaccine from those immunized with living virus. The former showed lesions with necrosis, whereas the latter did not.

SUMMARY

A study was made of the development of skin resistance and delayed hypersensitivity to vaccinia virus after immunization with inactivated vaccinia virus in rabbits. These vaccines are shown to increase skin resistance, and it is also shown that the development of this resistance is dependent upon the virus content of the vaccine. Skin resistance is shown to be related to the titre of circulating antibody rather than to the degree of delayed hypersensitivity. Possible reasons for conflicting reports in the literature on this subject are discussed.

An unusual skin reaction was seen in some animals when challenged with live virus after immunization with inactivated vaccine. The nature of these reactions is discussed.

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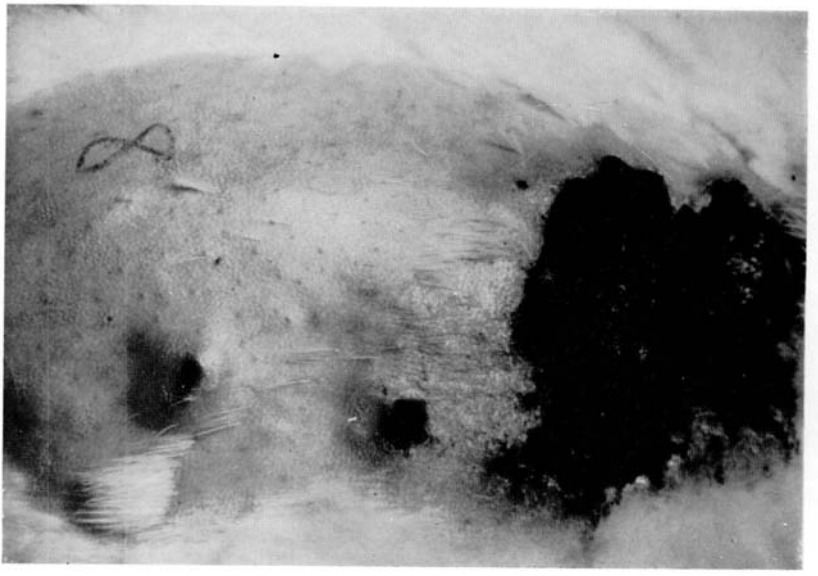

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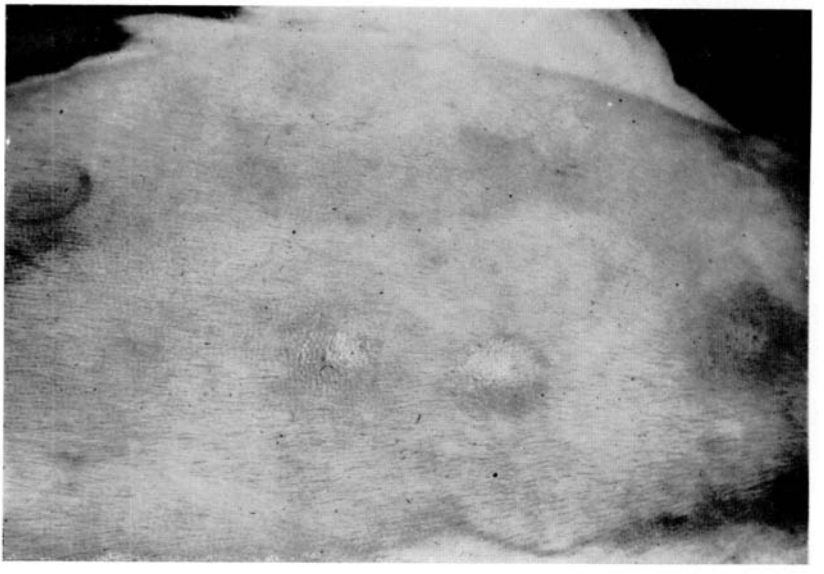

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REFERENCES

AMIES, C. R. (1961). Loss of immunogenic properties of vaccinia virus inactivated by formaldehyde. *Can. J. Microbiol.* **7**, 141.

ANDREWES, C. H., ELFORD, W. J. & NIVEN, J. S. F. (1948). Vaccinia and ectromelia in the mouse. *Br. J. exp. Path.* **29**, 329.

BEUNDERS, B. J. W., DRIESSEN, J. H. & VAN DEN HOEK, C. (1960). Clinical picture and serological response to vaccination with formalinized vaccinia virus followed by scarification with active vaccine in military personnel. *Arch. ges. Virusforsch.* **10**, 382.

BUSSEL, M. & MAYZNER, M. (1930). Etudes sur la vaccine formolée et sur la réaction variolique *C. r. Séanc. Soc. Biol.* **103**, 411.

COLLIER, L. H., MCCLEAN, D. & VALLET, L. (1955). The antigenicity of ultra-violet irradiated vaccinia virus. *J. Hyg., Camb.*, **53**, 513.

EHRENGUT, W. (1959). Erfahrungen mit Vakzineantigen. *Münch. med. Wschr.* **101**, 921.

KAPLAN, C. (1960). The antigenicity of γ-irradiated vaccinia virus. *J. Hyg., Camb.*, **58**, 391.

MCNEILL, T. A. (1965). The antibody response of rabbits to inactivated vaccinia virus. *J. Hyg., Camb.*, **63**, 525.

PARKER, R. F. & RIVERS, T. M. (1936). Immunological and chemical investigations of vaccine virus. III. Response of rabbits to inactive elementary bodies of vaccinia and to virus-free extracts of vaccine virus. *J. exp. Med.* **63**, 69.

RAMANARAO, A. V. (1962). The immunogenicity of inactivated vaccinia virus. *J. Path. Bact.* **84**, 367.

WEIL, A. J. & GALL, L. S. (1940). Studies on the immunization of rabbits with formalinized vaccine virus. *J. Immun.* **38**, 1.

EXPLANATION OF PLATE

(*a*) The back of a rabbit, showing a severe type of reaction following intradermal challenge with live vaccinia virus. Photograph taken 6 days after challenge.

(*b*) The back of a rabbit, showing a normal reaction to vaccinia virus. Photograph taken 6 days after challenge.

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