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The neutralization of pox viruses

II. Relationships between vaccinia, rabbitpox, cowpox and ectromelia

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INTRODUCTION

The serological relationships between members of the vaccinia subgroup of pox viruses have been the object of several investigations. Downie & Macdonald (1950) and Macdonald & Downie (1950), using complement fixation with soluble antigens and rabbit antisera, showed that the degree of fixation tended to be greatest in homologous systems but did not clearly differentiate the viruses. Complement-fixation inhibition tests using fowl antisera showed that cowpox and ectromelia were more closely related to each other than either was to variola or vaccinia. Similar conclusions were reached by Gispen (1955) using immuno-diffusion. Very little information is available concerning the cross-relationships of these viruses in neutralization tests. Downie & McCarthy (1950), using pock-neutralization, were able to demonstrate differences by cross-neutralization and after cross-absorptions, but these experiments did not reveal any obvious quantitative pattern, and, as the authors point out, interpretation of the cross-absorption experiments was difficult since the antisera were not of comparable potency. Appleyard & Westwood (1964), using a 50% pock neutralization end-point, have shown that the titre of an antivaccinia serum can be quite different not only when titrated against rabbitpox virus, but also when titrated against different strains of vaccinia. The greater precision now available with plaque methods makes it reasonable to re-investigate these viruses by cross-neutralization in the hope that more definite relationships can be established. This paper is concerned with these relationships determined in the HEp 2 cell-assay system.

MATERIALS AND METHODS

Virus strains. The vaccinia was as described previously (McNeill, 1965). Rabbitpox Utrecht strain, cowpox Brighton strain and ectromelia Mill Hill strain were supplied by Dr C. R. Madeley. Each virus was passed several times in HEp2 cells and suspensions were prepared for neutralization tests after partial purification by differential centrifugation and resuspension with ultrasonic vibration. Virus was diluted to a concentration of 10^5 pfu/ml. in McIlvaine's buffer 0·004 M pH 7·2, containing 20% skim-milk (Oxoid). These stock suspensions were stored in small volumes at $-70°$ C. For kinetic studies the stock suspension was used at a 1/2 dilution and for neutralizing antibody titrations at 1/100, each dilution being made in 0·004 M McIlvaine's buffer containing 20% skim-milk.

Virus titrations and titrations of neutralizing antibody for each virus were performed by the method previously described for vaccinia (McNeill, 1965) with two modifications—(i) the antiserum in the overlay medium was homologous for the virus being titrated, and (ii) on account of their minuteness ectromelia plaques were given an extra day to develop.

Kinetic studies were performed by the dilution method described in the previous paper (McNeill, 1968).

Antisera

The preparation of antivaccinia serum has been described previously (McNeill, 1965).

Anti-rabbitpox. Four rabbits were each inoculated with 50 pfu virus intraperitoneally (I.P.), and the following day with 1 ml. high titre anti-rabbitpox serum intravenously (serum supplied by Dr G. Appleyard). Three weeks later each rabbit was given 10^6 pfu I.P. Following this inoculation two of the rabbits developed a fatal infection. The two survivors received two further I.P. doses of 10^7 pfu at 2-week intervals after this. The animals were bled out 1 week after the last inoculation and their sera pooled.

Anti-cowpox. Two rabbits were each given 10^6 pfu virus I.P. and this was followed 1 month later by a booster inoculation of 10^7 pfu. The animals were bled out 2 weeks after the second inoculation and their sera pooled.

Anti-ectromelia. Two rabbits were each given a course of five I.P. inoculations of 1 ml. virus suspension containing 10^8 pfu/ml. at 2-weekly intervals. They were bled out 2 weeks after the last inoculation and their sera pooled. As ectromelia does not infect rabbits the inoculated material consisted of ground-up infected chorioallantoic membranes which was clarified by light centrifugation. No attempt was made to purify the virus any further, since it was hoped that the soluble antigens present in the extract would give an antigenic stimulus similar to that given by the other viruses which produce soluble antigens during the course of infection.

All sera were sterilized by Seitz filtration and stored at $-20°$ C.

Serum fractionation. Pools of 7 S and 19 S antibody were prepared from each antiserum by the method described in the previous paper (McNeill, 1968).

RESULTS

Antibody titrations

50 % *plaque reduction titres*

7 S and 19 S antibody fractions for each virus were titrated against each virus and the results are given in Table 1. Each value is the average of at least four separate determinations.

It can be seen that: (i) vaccinia and rabbitpox give very close cross-titration values, (ii) the titre obtained when cowpox antibody is titrated against vaccinia or rabbitpox is approximately one half of that when cowpox virus is used, (iii) when vaccinia, rabbitpox or cowpox antibody is titrated against ectromelia the

titre is approximately one tenth that for the homologous virus, (iv) the titre of ectromelia antibody is approximately doubled when titrated against vaccinia or rabbitpox compared with ectromelia, (v) these relationships hold for both 7 S and 19 S antibody.

Table 1. *Cross-neutralizations with 7 S and 19 S antibody*

	Antibody							
	Vaccinia		Rabbitpox		Cowpox		Ectromelia	
Virus	7 S	19 S	7 S	19 S	7 S	19 S	7 S	19 S
Vaccinia	500	76	3550	90	125	38	280	22
Rabbitpox	650	95	3620	118	115	23	236	15
Cowpox	220	26	2150	94	384	56	340	28
Ectromelia	44	5	385	9	31	7	140	8

Table 2. *Slopes of titration lines in cross-neutralization tests with 7 S and 19 S antibody*

	Antibody							
	Vaccinia		Rabbitpox		Cowpox		Ectromelia	
Virus	7 S	19 S	7 S	19 S	7 S	19 S	7 S	19 S
Vaccinia	60°	40°	43°	49°	52°	43°	42°	43°
Rabbitpox	44°	44°	57°	42°	46°	43°	44°	41°
Cowpox	54°	45°	48°	46°	57°	43°	46°	48°
Ectromelia	37°	35°	41°	40°	40°	38°	60°	40°

Slope of titration lines

The slopes of the line relating serum dilution to percent virus survival are given in Table 2. The slopes are measured as the angle between the titration line and the abscissa using points between 20 and 70 % virus survival. It can be seen (i) that the slope for 7 S antibody in homologous systems is steeper than in heterologous systems, and (ii) that with 19 S antibody there is no consistent difference between homologous and heterologous systems.

Virus survival in excess antibody

The persistent virus infectivity in the region of antibody concentration just before infectivity breakthrough was measured for each virus against 7 S and 19 S antibody for each using fine dilution steps. It was found that each 7 S antibody preparation neutralized each virus to the same general level with the exception of ectromelia virus, which showed a slightly higher level of survival than the other viruses with all 7 S antibodies including its own. With 19 S antibody the outstanding feature was that ectromelia gave considerably higher levels of surviving virus with all antibody including its own. This is shown in Fig. 1, where the levels have been plotted to allow for the quantitative differences given in Table 1. With the additional exception of rabbitpox, which gave a higher persistent

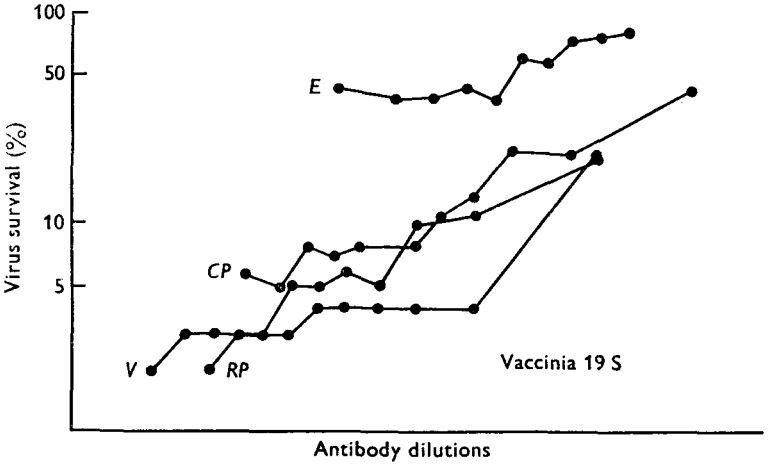

Fig. 1. Relationship between percentage virus survival and concentration of vaccinia 19 S antibody for ectromelia (*E*), cowpox (*CP*), vaccinia (*V*), and rabbitpox (*RP*). Results are plotted to take account of the titration differences shown in Table 1.

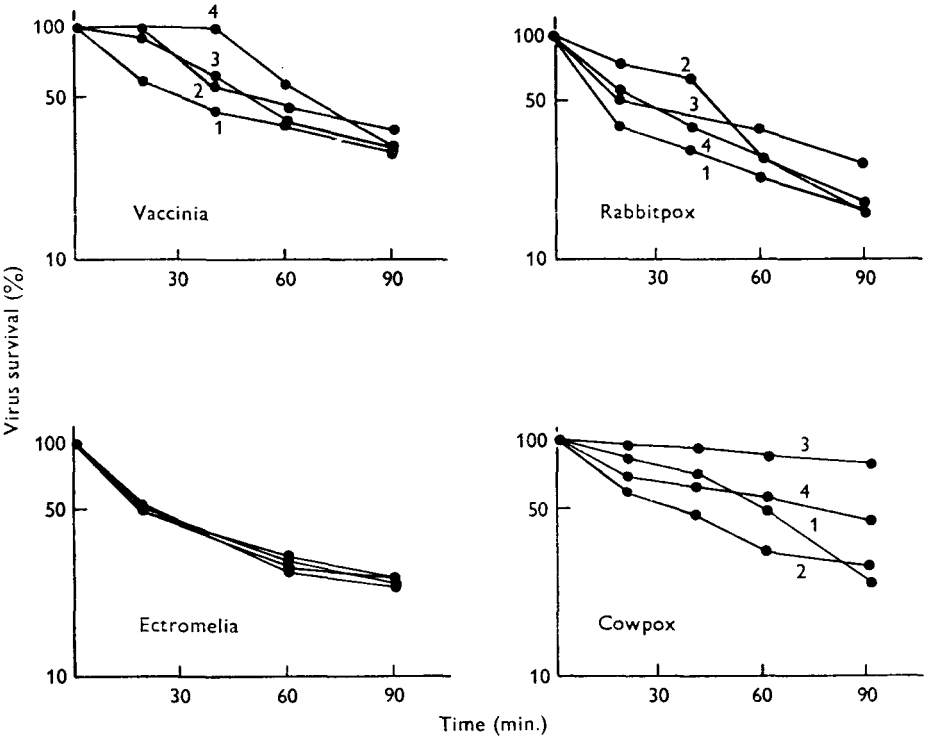

Fig. 2. Kinetics of neutralization of vaccinia, rabbitpox, ectromelia and cowpox viruses by 12 units of vaccinia 7 S (1), rabbitpox 7 S (2), cowpox 7 S (3), and ectromelia 7 S (4) at 4° C.

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fraction in excess cowpox 19 S antibody, all the other virus–antibody systems gave much the same levels of persistent infectivity.

Kinetics of neutralization

Figure 2 shows the kinetic slopes obtained in reactions with all 7 S antibodies. In each experiment the reaction temperature was 4° C. and antibody was used at a concentration of 12 units with reference to the titre of the antiserum with the particular virus, i.e. 12 units of vaccinia 7 S against vaccinia represented a 1/40 dilution, whereas 12 units of this serum against ectromelia represented a dilution

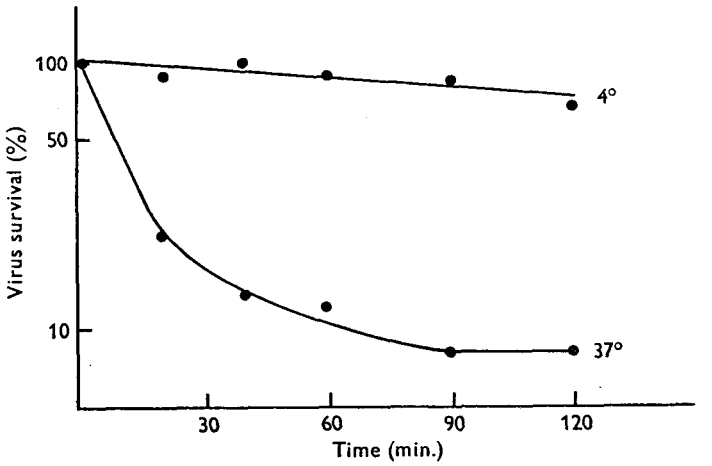

Fig. 3. Kinetics of cowpox neutralization by 12 units of anticowpox 7 S antibody at 4° C. and 37° C.

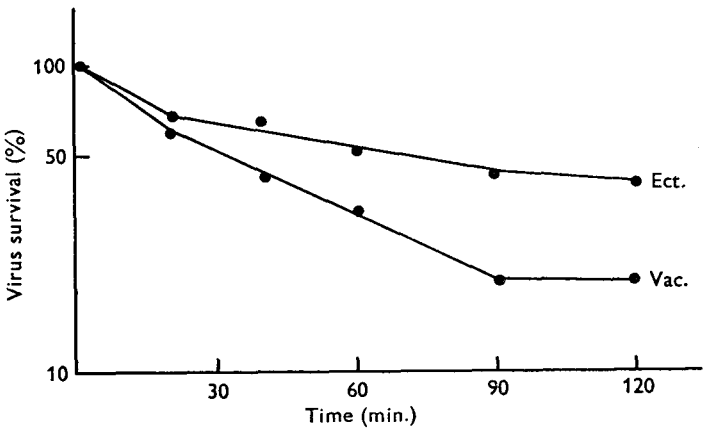

Fig. 4. Kinetics of neutralization of ectromelia and vaccinia viruses by 4 units of antiectromelia 19 S at 37° C.

of 1/4. It would be expected that the rate of neutralization be the same at equivalent concentrations of all antisera. However, this was only seen with ectromelia and the results with the other viruses were more complicated, each of these

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showing distinct shoulders on the kinetic curves with some antisera. It is noteworthy that cowpox was neutralized very poorly by its own antibody at 4° C., although when the reaction was carried out at 37° C. neutralization proceeded normally (Fig. 3).

In the light of the observation that ectromelia showed a high persistent fraction in excess 19 S antibody, kinetic studies were undertaken to determine if this was due to a prolonged initial delay in the neutralization reaction or to a normal initial reaction leading to a high persistent fraction. Figure 4 shows the kinetics of ectromelia and vaccinia neutralization by four units of ectromelia 19 S antibody, and this demonstrates that the latter alternative is correct.

DISCUSSION

These results have confirmed the very close immunological relationship of the viruses concerned and in addition have revealed quite precise relationships in cross-titration values. Rabbitpox and vaccinia were indistinguishable on cross-titrations, whereas cowpox and to a greater extent ectromelia are clearly separable from each other and from rabbitpox and vaccinia. It must be emphasized that these relationships can only apply to the particular strains studied using the HEp 2 cell assay system. Appleyard & Westwood (1964) found clearly distinguishable cross-titration differences between rabbitpox and vaccinia using the chorioallantoic membrane as assay system, so it is obvious that no general interpretation of these differences is justified on the basis of results from a single assay system with single strains of virus. The results of vaccinia neutralization in HEp 2 and monkey kidney described in the previous paper emphasize the same point. The reduction in titration slope noted by Appleyard & Westwood (1964) when vaccinia and rabbitpox viruses were titrated against heterologous antisera has been shown to apply to all four viruses in heterologous systems with 7 S antibody only. On the basis of the hypothesis of antibody competition proposed in the previous paper (McNeill, 1968) a reduction in titration slope indicates that the resultant effect of competition is proceeding in favour of neutralization. In the rabbitpox-vaccinia systems, where the titration end-points and presumably the number of antibody molecules involved are the same, the reduction in slope in heterologous systems is most simply explained by assuming that the antigenic sites for neutralization (N) of the two viruses are identical, and that the difference lies in the avidity of interfering (I) antibodies. This difference must be slight, and could be due to minor variations in the structure of I antigens or to the same I antigen being presented at the virus surface in a slightly different manner. The cross-neutralization kinetic results shown in Fig. 2 are also consistent with the conclusion that the viruses possess identical N antigens. The most straightforward results were with ectromelia, which gave identical kinetics with 12 units of all four antisera. A marked initial delay in the neutralization of vaccinia was seen with heterologous antibody. However, the results with rabbitpox and cowpox clearly show that this delay is not confined to heterologous systems, and neither is it a consistent feature of all reactions with a particular virus. It could be due to

dissociation of *N* antibody on dilution of the virus-antibody mixtures. Lafferty (1963) has shown that this instability on dilution is very marked with monovalent antibody fragments and has proposed that stabilization occurs when the antibody molecule has made a bivalent attachment to the virus.

It has been shown that ectromelia virus gives a high persistent fraction with the 19 S antibody of all four viruses (Fig. 1), and that this is not due to any delay in the initial rate of neutralization (Fig. 5). This effect is therefore a property of 19 S antibody reacting with ectromelia virus in particular and could indicate that the *N* sites of ectromelia are relatively inaccessible and therefore attachment of *N* antibody is more easily blocked by interfering antibody, especially when it is of macroglobulin type.

SUMMARY

Plaque neutralization on HEp 2 cells was used to determine the relationship between vaccinia, rabbitpox, cowpox and ectromelia using both 7 S and 19 S antibody. It was shown that for the strains of virus used vaccinia and rabbitpox were very similar but cowpox and particularly ectromelia were clearly distinguishable. The cross-titration relationships were the same for 7 S and 19 S antibody.

The titration slopes and kinetics of cross-neutralizations were interpreted on the basis of a hypothesis of antibody competition put forward in the preceding paper. It was tentatively concluded that these four viruses share a common critical antigen for neutralization and that differences in the structure or presentation of neighbouring antigens could give rise to some of the observed effects.

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