J. Hyg., Camb. (1968), **66**, 541
Printed in Great Britain

The neutralization of pox viruses

I. Evidence for antibody interference

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(*Received* 5 *March* 1968)

INTRODUCTION

Inactivated vaccinia virus can stimulate rabbits to develop neutralizing antibody and delayed hypersensitivity (McNeill, 1965, 1966), but how these factors are related to protective immunity is not known. Appleyard & Westwood (1964) have shown that the degree of protection against rabbitpox bears little relationship to levels of neutralizing antibody. Therefore either some other factor such as delayed hypersensitivity is more important, or titres of antibody measured by pock neutralization do not directly reflect its protective quality. With the latter possibility in mind the neutralization of vaccinia virus was investigated in more detail.

When a constant quantity of virus is added to a series of antibody dilutions the resulting titration profile shows two portions: (i) a persistent relatively low level of virus infectivity even in gross antibody excess, and (ii) a zone of infectivity breakthrough—the titration slope. The persistent fraction has been variously attributed to non-hereditary differences in the antigenic constitution of the particles (Dulbecco, Vogt & Strickland, 1956), dissociation on the basis of mass-law equilibria (Fazekas de St Groth & Reid, 1958), potentially infectious virus-antibody complexes (Bradish, Farley & Ferrier, 1962), non-avid antibody interfering with avid neutralizing antibody (Lafferty, 1963), and presence of virus aggregates in which some particles are protected from antibody (Wallis & Melnick, 1967). It has been generally thought that the titration slope is the result of limiting concentration of neutralizing antibody molecules whose uniformity of function is assumed.

It is the purpose of this paper to show that for pox viruses, at least, the degree of neutralization is not simply a direct relationship between virus and one type of antibody, but is the resultant effect of competing antibodies.

MATERIALS AND METHODS

The vaccinia virus, rabbit antiserum, and the methods of infectivity and neutralizing antibody titrations were as previously described (McNeill, 1965). In some experiments excess antibody was removed before assay by two cycles of centrifugation at 10,000 rev./min. for 60 min. in $3 \times \frac{1}{2}$ cm. tubes with resuspension into McIlvaine's buffer. This is referred to as washed virus. In one experiment neutralization was assayed in monkey kidney cultures in parallel with HEp 2

cell cultures. Second pass rhesus kidney was grown in 1 oz. flat bottles with Hanks's lactalbumin containing 2 % calf serum. These monolayers were inoculated for plaque assay in exactly the same way as the HEp 2 cell monolayers.

Neutralization kinetics. Stock virus (5×10^4 p.f.u./ml. in 0·004 M McIlvaine's buffer, pH 7·2, containing 20 % skim-milk (Oxoid)) and dilutions of antibody in McIlvaine's buffer were brought to a suitable temperature and mixed. The reaction was stopped at various times after mixing by making a 1/50 dilution into McIlvaine's buffer + 20 % skim-milk. When all samples were collected, residual infectivity was assayed by the standard plaque method, and expressed as the percentage of a control mixture of virus and buffer.

Serum fractionation. Antivaccinia rabbit serum was fractionated on Sephadex G-200 using a filled K 25/45 column (Pharmacia Fine Chemicals, Uppsala) and 0·01 M phosphate buffer pH 7·2 as eluent. Pools of 19 S (mercaptoethanol sensitive) and 7 S (mercaptoethanol resistant) antibody were made from fractions showing the highest neutralizing antibody activity in the two antibody elution peaks. Neither the original serum nor the fractions were heat inactivated. Sera and fractions were stored in small amounts at −20° C.

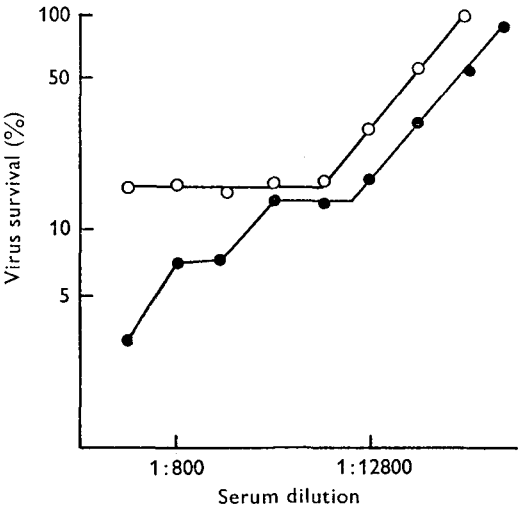

Fig. 1. Titration profile of an antiserum and the effect on this of washing the neutralized virus before assay. Filled circles, unwashed virus; open circles, washed virus.

RESULTS

Titration profile

In Fig. 1 the titration profiles are shown for a hyperimmune serum when the neutralized virus was inoculated either washed or unwashed. In the unwashed series there is a stepwise increase in percentage virus survival to a point of linear breakthrough which gives the titration slope. Washing resulted in a higher but constant level of infectivity in antibody excess, and a slight shift to the left of the titration slope. The stepwise increase in virus survival with unwashed mixtures is seen more clearly in Fig. 2, which shows the titration profiles of 7 S and 19 S

antibody. This figure shows the composite results of several experiments in which very small dilution steps were used. These dilution steps were made from starting dilutions of the antibody pools in the following series of antibody : buffer ratios.

Antibody (ml.)	0·21	0·19	0·17	...	0·05
Buffer (ml.)	0·09	0·11	0·13	...	0·25

In order to facilitate comparison of the profiles the results for each type of antibody have been superimposed without regard to the actual starting dilution of each pool. It can be seen that 7 S and 19 S antibody differ only in the steepness of the titration slope.

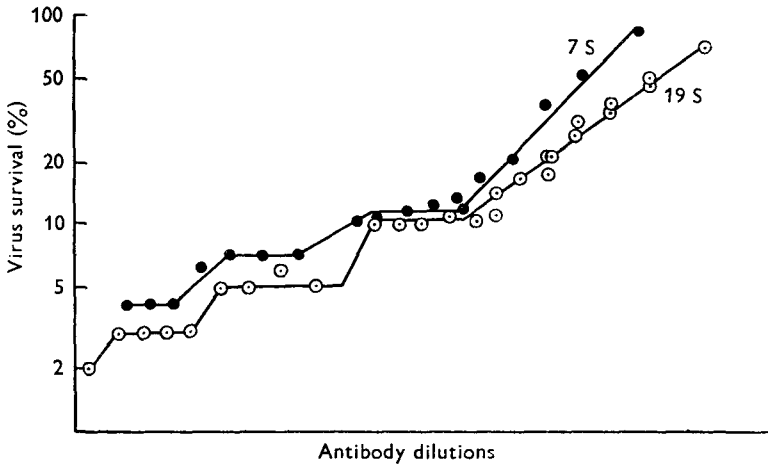

Fig. 2. Titration profiles of 7 S and 19 S antibody.

Table 1. *Percentage virus survival in a series of serum dilutions and following each of two absorptions of these dilutions*

Antibody dilutions	Percentage virus survival		
	Original dilutions	First absorption*	Second absorption*
1/4000	7	6	14
1/8000	7	8	32
1/16,000	8	11	65
1/32,000	15	35	89
1/64,000	39	51	96
50% plaque neutralization titre*	1/80,000	1/110,000	1/52,000

* N.B. Allowance has been made for a twofold dilution of antibody with each absorption

Titration slope—antibody competition

Experiment 1. The supernatant of a virus–antibody mixture from which virus has been removed by centrifugation will neutralize fresh virus to the same percentage as the original virus. The results of the following experiment illustrate this point. A series of twofold antibody dilutions were each mixed with 300 pfu of virus and incubated for 2 hr at 37° C. One half of each mixture was kept for virus assay and

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the other centrifuged for 1 hr. at 10,000 rev./min. in $3 \times \frac{1}{2}$ cm. tubes. An equal volume of virus was added to each supernate and reincubated for 2 hr. at 37° C., after which the whole process was repeated. Table 1 gives the percentage virus survival at each dilution for each of these three series of neutralizations.

Experiment 2. When additional antibody is added to a series of completed virus–antibody mixtures (*without washing the virus before the addition*) the percentage of surviving virus is not diminished. This is shown by the following experiment. Two series of twofold dilutions of high titre antiserum from 1/8000 to 1/128,000 were made and an equal volume of virus (300 pfu) added to each dilution. After 2 hr. at 37° C. an additional volume of 1/4000 antiserum was added to each tube in one series and the same volume of buffer to each tube in the other series. These mixtures were incubated for a further 2 hr. at 37° C. and the virus in each was washed by two cycles of centrifugation + resuspension in McIlvaine's buffer. This washing is essential, since excess unbound antibody can have an effect if inoculated with virus into the assay system as shown in Fig. 1. The results are shown in Table 2.

Table 2. *Percentage virus survival in a series of antibody dilutions when excess antibody is added after the first reaction*

Serum dilution	% virus survival 2nd incubation with buffer	% virus survival 2nd incubation with excess antibody
1/8000	16	17
1/16,000	32	33
1/32,000	57	45
1/64,000	79	77
1/128,000	99	89
Buffer only	100	15

Kinetics of neutralization

In these experiments antibody concentrations are expressed in arbitrary units — one unit being contained in that dilution of serum giving 50 % plaque neutralization.

7 S *and* 19 S *antibody*. The kinetic results for 3, 6 and 12 units of each type of antibody at 4° C. are shown in Fig. 3. It can be seen that 7 S neutralizes more rapidly than 19 S at 'equivalent' concentration; that with both types there is a change in rate during the course of neutralization; and that the increase in rate with increasing concentration is greater for 7 S than it is for 19 S. Figure 4 shows *that increasing the temperature also increases the rate of neutralization by 7 S* more than that by 19 S.

Different assay systems. Figure 5 shows the kinetic slopes of virus neutralization with 12 units of antibody at 37° C. when the same reaction mixtures were assayed in monkey kidney (M.K.) and HEp 2 cells. It is clear that the rate of neutralization is apparently much slower when assayed in M.K. Other experiments showed that the level of persistent infectivity in 100 units of antibody after 4 hr. at 37° C. was much higher in M.K. than in HEp 2 (30 % compared with 4 %). The mean

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50 % plaque neutralization titre for the serum was 1/20,000 in HEp 2 and 1/5000 in M.K. The virus control counts showed that the HEp 2 cells were three times as sensitive to virus as the M.K.

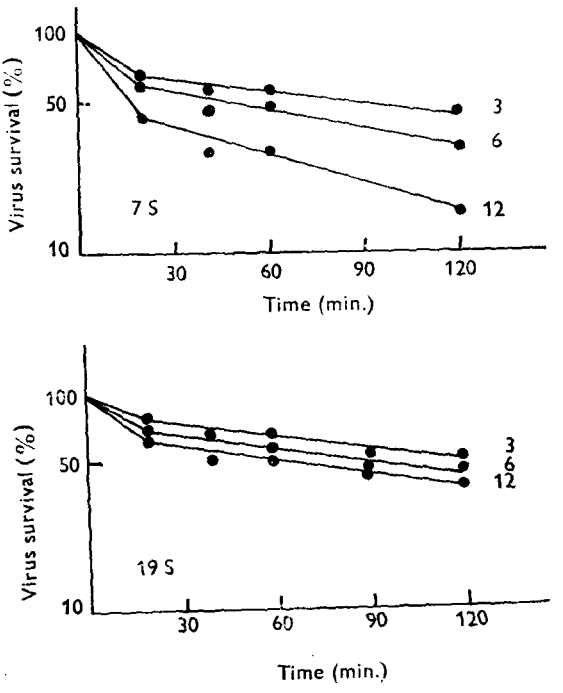

Fig. 3

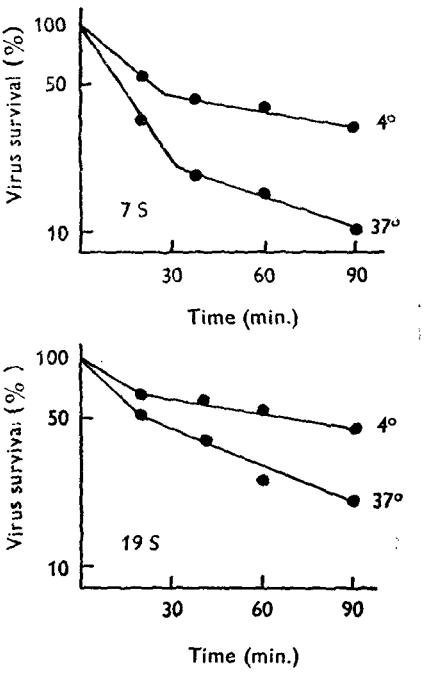

Fig. 4

Fig. 3. The kinetics of neutralization of vaccinia with 7 S and 19 S antibody at 4° C. Effect of antibody concentration (3, 6 and 12 units).

Fig. 4. Kinetics of neutralization of vaccinia with 7 S and 19 S antibody. Effect of temperature (4 and 37° C.).

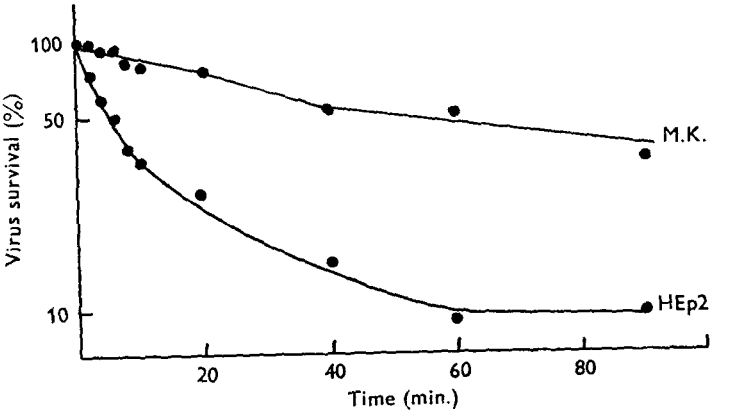

Fig. 5. Kinetics of neutralization. The effect of assaying the same reaction mixtures in HEp 2 cells and monkey kidney.

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DISCUSSION

The titration slope

Experiment 1 shows that antibody is present in excess at dilutions corresponding to the titration slope. Experiment 2 shows that a partially neutralized virus suspension on the titration slope cannot be further neutralized by the addition of more antibody. The only explanation of these findings is that such unneutralized virus has become protected from neutralizing antibody. The percentage virus survival at any serum dilution must be the resultant of virus–antibody reactions having opposite effects. However, since both types of antibody would be present in the same proportion at all serum dilutions it is difficult to explain the sharp transition in percentage virus survival which gives the titration slope. It could be postulated either (*a*) that neutralizing (*N*) antibody is much less avid than interfering (*I*) antibody, or (*b*) that a third type of antibody is involved—anti-interfering (*AI*)—which when combined with sites adjacent to an *I* site can block the attachment of *I* antibody thus leaving the *N* site open for *N* antibody. If the *AI* antibody reached limiting dilution first in a series of serum dilutions, further dilution would result in virus neutralization becoming progressively more dependent upon straightforward competition between *N* and *I* antibody, thus giving the titration slope. The steepness of the slope would depend upon the $(N+AI)/I$ ratio at the appropriate dilutions both in terms of relative concentrations and quality of the various antibody molecules.

Such a model is necessarily speculative but is at least consistent with the observations that when 7 S and 19 S antibody are compared the increase in neutralization rate with increasing concentration of antibody (Fig. 3) and increasing temperature of reaction (Fig. 4) is less for 19 S than 7 S. This effect could be simply explained by the interference hypothesis on the basis of molecular size if the 19 S *AI* molecules not only block the attachment of *I* antibody, but being large molecules also can interfere with the attachment of *N* antibody.

The persistent fraction

A consequence of the interference hypothesis is that even at high antibody concentrations there will be a proportion of virus particles protected by *I* antibody. This would contribute to the persistent fraction for which various explanations have been listed in the introduction. Wallis & Melnick (1967) have proposed that the persistent fraction for several viruses, including vaccinia, is due to the presence of virus aggregates. This may account for it in part, but cannot account for it entirely, since that would be inconsistent with several features reported by Lafferty (1963): (*a*) addition of excess antibody to *washed* virus in the persistent fraction further decreases infectivity, (*b*) the same virus preparations against different antisera can show markedly different persistent levels, and (*c*) the persistent fraction can be greatly influenced by the assay system. If part of the persistent fraction depended upon the resultant effect of competing antibodies on and around critical neutralization sites it would be expected to vary with different antisera. The cell-dependent aspect of the persistent fraction is particularly

interesting, and Lafferty (1963) suggested that it was due to antibody recognized as neutralizing in one system but not in another, such antibody even protecting the virus from antibody having a neutralizing effect for the second system.

A simpler explanation using the interference hypothesis would be that to ensure neutralization some cells require more virus *N* sites to be inactivated than other cells. The greater the number of *N* sites which have to be neutralized the greater will be the opportunity for *I* antibody to protect the virus and one manifestation of this will be a higher persistent fraction. Therefore it could be predicted that when one assay system required neutralization of more *N* sites than another it will show apparently slower neutralization kinetics, a higher persistent fraction and a lower 50 % plaque reduction neutralization titre for a serum. The results of parallel assays of virus–antibody mixtures in HEp 2 and M.K. (Fig. 5) are consistent with these predictions.

The phenomenon of antibody interference is obviously relevant to the value of an antibody response in protection against virus infection, since it would be expected that the protective quality of the response would depend on the proportions and relative avidity of the neutralizing and interfering antibodies as well as on the absolute concentration of neutralizing antibody. This phenomenon may at least partly explain why some authors have observed little correlation between levels of neutralizing antibody and the degree of protection against infection.

SUMMARY

Evidence was presented to support a hypothesis that competition between antibody molecules can be an important factor in vaccinia virus neutralization.

It has been shown that there is little difference in the virus neutralizing properties of 7 S and 19 S antibody and it has been further postulated that in the context of protective immunity the proportions of antibodies having neutralizing and interfering effects is likely to be more important than the absolute concentration of neutralizing antibody or the type of immunoglobulin in which it is present.

I wish to thank Mr Mervyn Killen for excellent technical assistance. I would also like to thank Prof. K. B. Fraser for some very helpful discussions during the preparation of the manuscript.

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