

SPECIAL ARTICLE

Insights into neutralization of animal viruses gained from study of influenza virus

INTRODUCTION

It has long been known that the binding of antibodies to viruses can result in a loss of infectivity, or neutralization, but little is understood of the mechanism or mechanisms of this process. This is probably because neutralization is a multifactorial phenomenon depending upon the nature of the virus itself, the particular antigenic site involved, the isotype of immunoglobulin and the ratio of virus to immunoglobulin (see below). Thus not only is it likely that neutralization of one virus will differ from another but that changing the circumstances of neutralization can change the mechanism itself. To give coherence to the topic we are concentrating this review on one virus, influenza type A which is itself well studied and reasonably well understood [1–3]. Reviews of the older literature can be found in references 4 to 7.

INITIAL STAGES OF INFLUENZA VIRUS INFECTION

Any of the early stages of infection can in theory be the target of neutralization of a virus and thus it is a prerequisite to the understanding of neutralization to have a complete picture of these virus-cell interactions. An influenza virus particle (Fig. 1) consists of a nucleocapsid structure surrounded by a lipid envelope derived as the nucleocapsid buds through the plasma membrane. Both these structures protect the viral genome from damage by nucleases. Embedded in the envelope are haemagglutinin (HA) proteins (about 1000/particle [8]) which function as ligands and attach the virus to specific cellular receptors. Fig. 2 shows five stages of the early interactions of influenza virus with a susceptible target cell. The HA binds to a carbohydrate moiety, *N*-acetyl neuraminic acid (NANA) (sialic acid). This is almost certainly attached to a protein as NANA-bearing glycolipids are short molecules, buried beneath the longer cell surface proteins and relatively inaccessible to virus particles. Virus is then taken up into a vesicle by endocytosis. A multi-stage process of 'uncoating' follows whereby the viral genome becomes functional and able to direct virus multiplication. This commences when a proton pump in the membrane acidifies the internal environment of the vesicle, causing the HA to undergo a conformational change which leads to primary uncoating – fusion of the viral and vesicle membranes and entry into the cytoplasm proper of the nucleocapsid [10–12]. Details of secondary uncoating can only be surmised but there have to be changes in nucleocapsid such as loss of proteins and/or relaxation of structure so that the viral genome can be exposed sufficiently to be expressed.

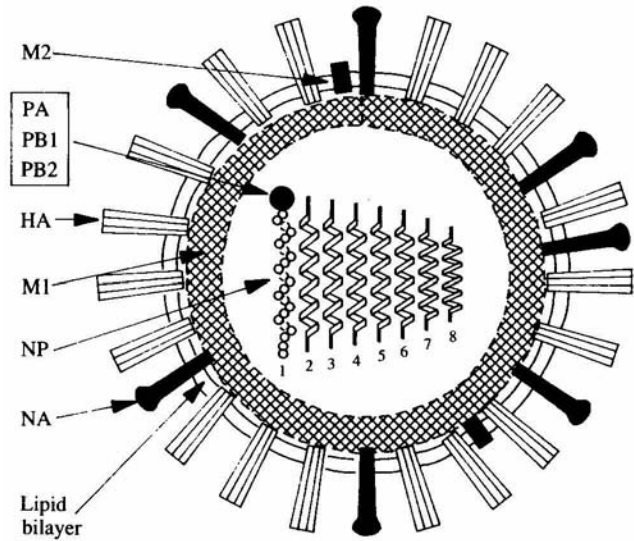


Fig. 1. Diagram (adapted from reference 56) of the structure of an influenza virus particle (not to scale). The inner core structure contains eight RNA segments each coated with nucleoprotein (NP) and containing one transcriptase complex composed of PA, PB1 and PB2, and surrounded by a layer of Matrix (M) 1 protein. Around this is a lipid membrane through which are inserted about 1000 haemagglutinin (HA) trimers, 400 neuraminidase (NA) and around 20 matrix (M) 2 molecules.

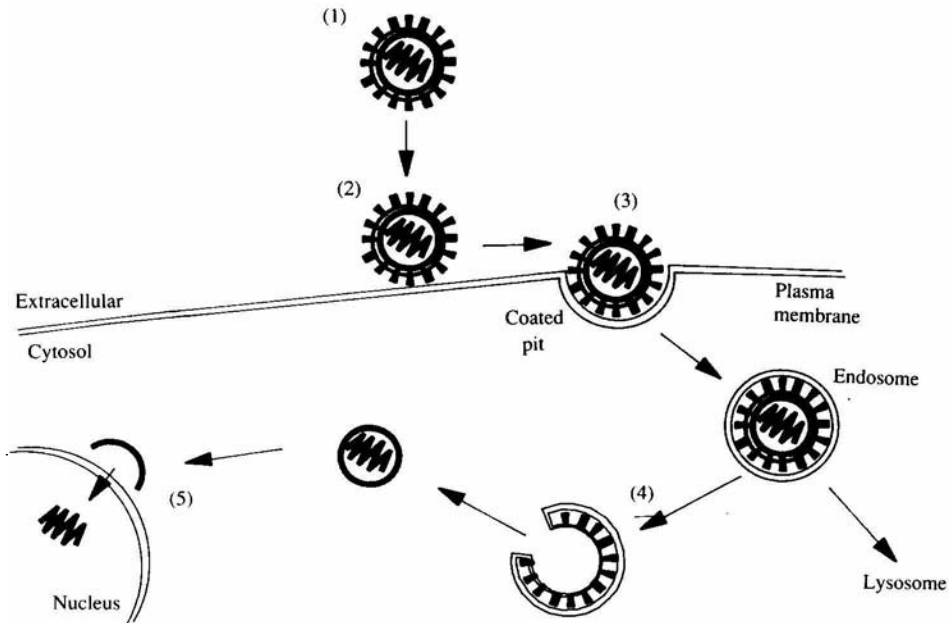


Fig. 2. A schematic representation of the pathway of influenza virus entry into cells. The stages at which neutralizing antibody may inhibit this process are discussed in the text.

The latter takes place in the cell nucleus and hence the nucleocapsid must be transported to that location.

Other viruses enter cells with only minor variations upon the above theme. Initially they all interact very specifically with a receptor on the cell surface which is often, but not always, a protein. Viruses frequently utilise molecules which perform some homeostatic function [9] and are therefore an obligatory feature of the target cell. The reaction takes place between complementary sequences on the virus (the attachment site) and the cell receptor unit. These are invariant presumably since sequence changes would deny the recognition process upon which infection ultimately depends. From there many enveloped and non-enveloped viruses follow a pathway similar to influenza virus and enter the cell by endocytosis. Other enveloped viruses (e.g. HIV [13], Newcastle Disease Virus [14]) enter cells by fusing with the plasma membrane itself at neutral pH.

MECHANISMS OF NEUTRALIZATION OF INFLUENZA VIRUS

Much of the following data come from our own laboratory. In the past we and others have varied virus concentration according to the demands of different technical procedures, often over many orders of magnitude which has made comparisons uncertain. To avoid the problem we have more recently used a constant virus concentration (500 units/ml) and varied only the antibody concentration.

The role of aggregation in neutralization

Aggregation of influenza virus by antibody has been determined by counting the aggregates and the number of particles contained therein under the EM. All isotypes tested (IgG, A and M) caused aggregation and all gave a similar pattern of aggregation [15, 16]. Fig. 3 shows that as the IgG concentration increased, aggregation increased and the infectivity of the virus fell. It was calculated that at the point of maximum aggregation the potential loss of infectivity due to aggregation alone was between 65 and 80%, but this did not fully account for the 99% neutralization observed. With further increase in IgG concentration aggregation decreased until the virus became as disperse as the non-neutralized controls. There was no concomitant return of virus infectivity, demonstrating that neutralization could not be due to aggregation alone, and that other mechanisms must be operating.

Caution is needed in the interpretation of aggregation data as in some virus-antibody mixtures the rise in aggregation *preceded* loss of infectivity. This may have been caused by disruption of the aggregates, shifting the curve to the right, or the process of drying the samples onto the EM grid may have enhanced aggregation by concentrating the virus and shifted the curve to the left. Analysis of the aggregates in solution (as with poliovirus: [17]) is needed to resolve the practical implications of this issue.

Inhibition of attachment of virus to cells by neutralizing antibody

Neutralizing IgG mabs directed to sites A, B and D in the influenza HA [18] all inhibited attachment and this correlated more closely with the loss of infectivity than did aggregation. Reduction in attachment to tracheal epithelial (Fig. 4) and

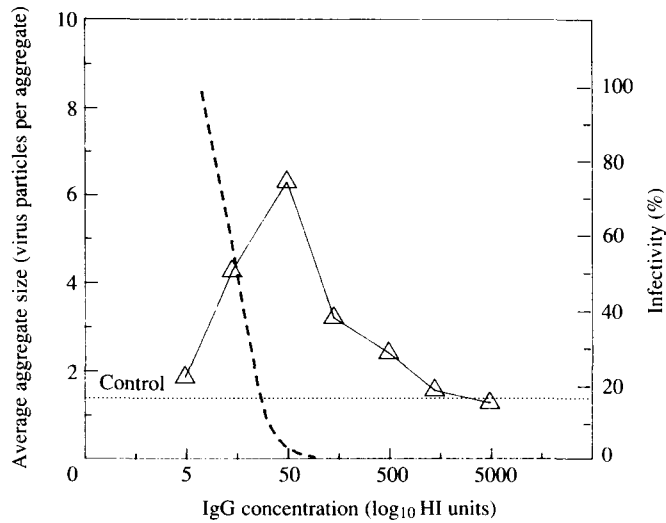


Fig. 3. Aggregation of FPV/R, a type A influenza virus (A/fowl plague virus/Rostock/1/34: H7N1) by a mouse monoclonal IgG2a (HC2) directed against the HA. The antibody concentration is expressed in haemagglutinin-inhibiting (HI) units defined by that amount which will inhibit the agglutination of erythrocytes by four HA units of virus. Virus (500 HA units/ml) and IgG were incubated together for 1 h at 25 °C and the amount of aggregation determined by electron microscopy. This is expressed as the average number of virions per aggregate ($-\Delta-$) and compared to non-neutralized controls (\cdots). Also shown is the extent of neutralization ($---$). From reference 16.

BHK cells reached a maximum of approximately 60 and 40% respectively but at no point did this account fully for the >99.9% neutralization observed [16]. Increasing the IgG concentration further resulted in a return to similar amounts of attachment as found with infectious virus. This agrees with earlier work using a single saturating IgG concentration where attachment to various cultured cells of chicken, mouse and human origin was unaffected [1, 19, 20]. Attachment of virus neutralized by monomeric IgA was also unimpeded [19]. In contrast to BHK and tracheal epithelial cells, attachment of IgG-neutralized virus to chicken erythrocytes was reduced by a greater degree and attachment remained uniformly low even at the highest IgG concentrations. Thus the target cell can be important when investigating the neutralization and differences between tracheal epithelial cells and erythrocytes demonstrate that the latter is a poor model of the *in vivo* situation. However, IgG-neutralized virus failed to attach to a B cell lymphoma cell line [21] emphasizing how each situation has to be assessed individually.

What lies behind these observations? Influenza virus attaches to sialic acid moieties. On erythrocytes the most abundant sialylated protein is glycophorin A which extends only a short distance, about 5 nm, from the cell surface [22, 23]. It may be that IgG molecules (about 14 nm) are sufficient sterically to hinder attachment of the virus to the erythrocyte cell but not to a putatively longer receptor (> 14 nm) on tracheal epithelial or BHK cells. Reduction in attachment is not due to aggregation of virus because electron microscopy shows that virus aggregates are able to attach to the cells. Reduction in attachment may be related to the density of neutralizing antibodies on the virus surface: thus at sub-

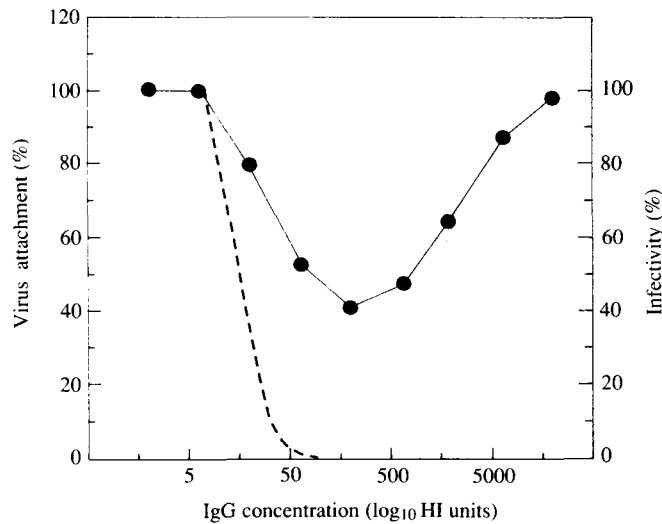


Fig. 4. Neutralization by HC2 of influenza A FPV/R and attachment of virus to mouse tracheal epithelial cells (—●—). Also shown is the neutralization profile (---). For other details see legend to Fig. 3. From reference 16.

saturation concentrations IgG molecules are loosely packed on the virus surface and have freedom to rotate [24–26] which prevents attachment of neutralized virus to cells. Increasing the IgG concentration results in a closer packing of IgG molecules on the virus surface and reduces IgG mobility. In turn this facilitates the attachment of the virus to cell receptors which reach between the IgG molecules.

What then is the significance to neutralization of aggregation and inhibition of attachment that occurs at lower IgG to virus ratios? At least for the monoclonal IgG HC61 the loss of infectivity due to the combined effects of aggregation and inhibition of attachment match very closely the amount of neutralization observed (Fig. 5). However for other mabs aggregation preceded neutralization suggesting that it was either an artifact of electron microscopy or that aggregates were easily dispersed by normal pipetting procedures, as discussed above.

With antibody of the IgM class, attachment of influenza virus to the three cell types tested (BHK, tracheal epithelial and chicken erythrocytes) was reduced and there was no return of attachment as the IgM concentration was increased [15]. The large size of the IgM molecules probably sterically hinders the attachment to all the cell types although some virus can still attach even at the highest IgM concentrations used. Neutralization of influenza virus by a murine monoclonal IgA gave similar results. This antibody is polymeric but unlike human IgA which usually polymerizes as dimers the mouse IgA is a mixture of dimers, trimers and tetramers. The monoclonal IgA lacks the secretory component which is only acquired by the antibody during its passage into external secretions but there is good agreement with data using polyclonal rat IgA from bile which has a secretory component [19].

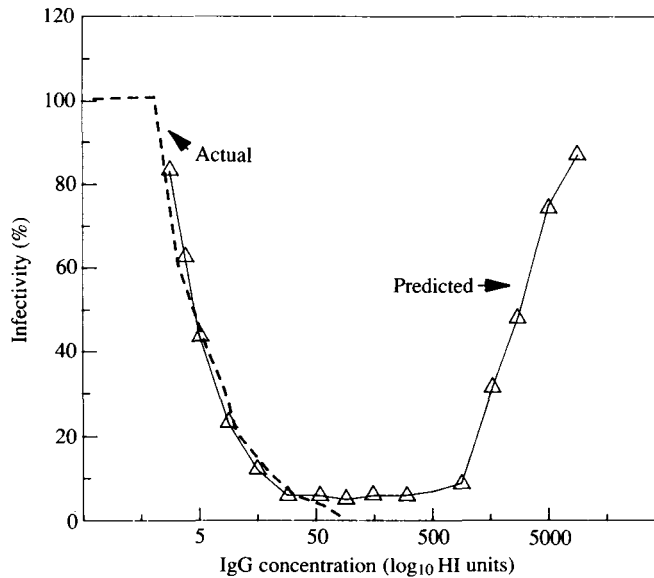


Fig. 5. Comparison of the actual neutralization of influenza A (FPV/R) by HC61 (---) with that calculated from the combined effects of aggregation and inhibition of virus attachment to BHK cells ($-\triangle-$). For other details see legend to Fig. 3. From reference 16.

Is internalization of virus prevented by neutralizing antibody?

Irrespective of the immunoglobulin isotype, some neutralized virus attached to cells. It is therefore important to determine whether or not such virus was being internalized. Pretreatment of cells with bacterial neuraminidase prevented attachment of infectious, IgG-, IgA- and IgM-neutralized virus indicating that all used sialic acid moieties as their receptors [15, 16]. Internalization of virus can be determined by incubating with neuraminidase *after* attachment has taken place: virus on the cell surface is detached while internalized virus is not. Therefore an increase in resistance to neuraminidase action with respect to time would indicate that internalization was occurring. Between 5 min and 60 min post-inoculation, resistance of non-neutralized virus to release by neuraminidase increased on both BHK and tracheal epithelial cells. A similar increase in resistance was found with IgG-neutralization irrespective of the concentration of antibody used, indicating that such virus was internalized. In contrast the majority of IgM- and IgA-neutralized virus was not internalized. However the data are not sufficiently precise to preclude the possibility that some internalization does take place.

Thus neutralization of influenza virus by the polymeric antibodies, IgM and IgA, appears to be due in part to inhibition of attachment to cells and in part to prevention of internalization of the virus that does manage to attach (Table 1). Virus is aggregated at certain ratios of immunoglobulin to virus but it is not clear that these aggregates are stable enough to contribute to neutralization.

IgG neutralization is more complex. With high IgG concentrations neutralized virus is not aggregated and attachment to BHK or tracheal epithelial cells was not inhibited at all (except on B-lymphoma cells [21]). This IgG-neutralized virus is

Table 1. Summary of some effects of neutralizing antibodies on influenza virus type A

	IgG		IgM		IgA*	
	[Low]	[High]	[Low] or [High]		[Low] or [High]	
Aggregation	Yes	No	Yes	No	Yes	No
Inhibition of attachment	Yes	No	Yes		Yes	
	50-60%		70-80%		70-90%	
Inhibition of internalization	No	No	Yes		Yes	

* Polymeric.

internalized by cells. The infectivity of the virus was neutralized by more than 99%, therefore under these conditions IgG-neutralization must involve a later, intracellular stage of infection. Neutralization at lower IgG concentrations is more complex: there is some inhibition of attachment but attached virus enters cells and is probably neutralized by the same mechanism as found with high IgG concentrations. It is not clear if virus-IgG aggregates are sufficiently stable to contribute significantly to the loss of infectivity.

'Intracellular' neutralization by IgG

Work on the intracellular mechanism of neutralization has shown that IgG-neutralized virus is uncoated, losing its lipid envelope in the cytoplasm [27]. This primary uncoating suggests that fusion of viral and endosomal membranes is occurring normally but this has yet to be verified directly. Virion RNA and associated proteins then migrate to the cell nucleus with kinetics indistinguishable from those of infectious virus but no primary transcription of the virus genome can be detected [27, 28]. The RNA of neutralized virus has been recovered from cell extracts and shown to be resistant to degradation by RNase whereas that from infectious virus was sensitive [28]. This suggests that IgG-neutralization blocks a secondary uncoating step which has to take place before the sub-viral core can transcribe the viral genome. The failure to initiate transcription is considered to be an epiphenomenon and we predict that if secondary uncoating could be achieved artificially, the transcriptase complex would be functional.

We suggest that the binding of neutralizing IgG to the HA spike induces or prevents a transmembrane signal which is needed to permit secondary uncoating of the virion core structure. It would seem likely that the major outer core protein, M1, which is adjacent to the COOH-terminal tail of the HA would be the protein most likely to be affected, although M2 may also be involved. We know that such cores artificially isolated from virus do not transcribe unless uncoated with detergent [28] and hence have the properties expected of cores from neutralized virus which are found in the nucleus.

How many molecules of IgG are needed to neutralize a virus particle?

To understand neutralization it is necessary to know if loss of infectivity is a single-hit process, resulting from the binding of one antibody molecule per virus particular or is multi-hit. Interpretation of earlier work [29, 30] is uncertain due

to the polyclonal nature of the antibody used. This has not been resolved by using monoclonal antibody.

Stoichiometric analysis

If every antibody molecule is neutralizing and every time one molecule binds to a virus particle it effects neutralization then Poissonian distribution states that when a virus population is neutralized by 63% there will be, on average, one antibody molecule per virus particle. However, experimentally it is found that there are 70 IgG molecules bound per influenza virus particle [8]. This was confirmed by electron microscopy showing, by colloidal gold labelling, the presence of IgG bound to virus at a dilution 100-fold less than that required to initiate neutralization. Further evidence, that at the commencement of neutralization there is not one but several molecules of monoclonal IgG bound per virus particle, comes from the enhancement of infectivity when such virus mixed with sub-neutralizing amounts of antibody are titrated on cells bearing Fc receptors [31, 32].

Kinetic analysis

Kinetics of neutralization (reviewed in 7) can be represented by equation (1):



where V_{inf} = infectious virus, V_{neut} = neutralized virus. This is a second-order reaction but it displays pseudo-first-order kinetics because neutralization is highly dependent on the concentration of antibody (ab) and less on virus concentration. The rate constant (k) can be calculated by plotting $\log_{10} V_t/V_0$ against time (t) according to equation (2):

$$\log_{10} \frac{V_t}{V_0} = \left(\frac{-k}{2.303D} \right)^t, \quad (2)$$

where V_t and V_0 are the infectivity titres at time t and 0 and D is the dilution factor or concentration of antibody. Fig. 6 shows a plot for the neutralization of influenza A virus by monoclonal IgG and the derived rate constant, k .

The number of antibody molecules required for neutralization to occur can be calculated from equation (3):

$$\log_{10} \frac{V_t}{V_0} = 1 - (1 - e^{-kt/D})^n, \quad (3)$$

where n is the number of antibody 'hits' required for neutralization; other symbols are as above. If $n = 1$ (i.e. single hit) then a plot of $\log V_t/V_0$ with respect to time gives a straight line and infectivity falls immediately. If n is greater than 1 (i.e. multi-hit) then a curve results and there is an initial lag period where little or no loss of infectivity occurs. For influenza A virus the kinetic data clearly show a single-hit mechanism (Fig. 6). The single-hit nature of the mechanism was further confirmed by varying the IgG concentration and comparing the predicted and actual amounts of neutralization (Fig. 7). These data confirm the conclusions of earlier work despite its use of heterogeneous polyclonal antibody [29].

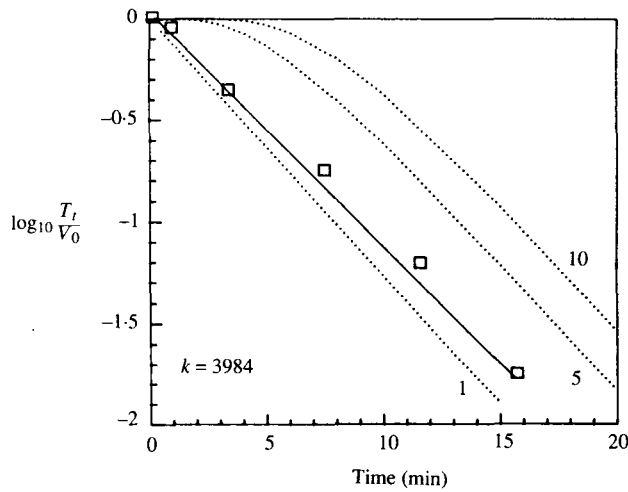


Fig. 6. Comparison of the observed rate of neutralization of influenza A (FPV/R) by HC2 (12 $\mu\text{g/ml}$; 77 μM) (\square) with that calculated when 1, 5 or 10 'hits' are required for neutralization (\cdots): see text for details. The rate constant (K) was calculated as described in the text.

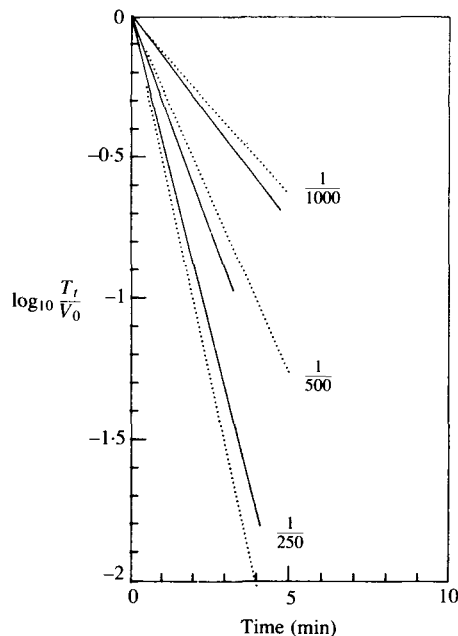


Fig. 7. Comparison of the actual (—) and predicted (\cdots) rates of neutralization of influenza A (FPV/R) by HC2 when different concentrations of IgG are used.

From the limited data so far available it seems that neutralization kinetics of IgM and IgG differ fundamentally. When influenza virus is incubated with IgM there is a significant lag of about 4 min before neutralization occurs suggesting that it follows multi-hit kinetics (M. C. Outlaw and N. J. Dimmock, unpublished

data). This is consistent with biochemical data showing that IgM neutralizes by inhibition of virus attachment to cells and the internalization of virus that does attach (see above) and with EM data showing that IgM binds to virus at a dilution approximately 100-fold greater than that necessary to elicit neutralization [33].

CRITICAL SITE MODEL OF IgG-NEUTRALIZATION

Data outlined above show that IgG-neutralization is a single-hit process and yet, at a dilution of antibody which causes 63% neutralization, there are on average 70 molecules of IgG bound per virus particle [8]. These apparently contradictory data can be resolved by invoking a critical site model which states that there are relevant (or critical) and irrelevant sites on the virus particle. Antigenically these sites are identical but differ functionally in that the binding of antibody to a relevant site leads to neutralization whereas binding to an irrelevant site does not. We propose that HA spikes differ only in the contact they make with the virus core: it is only through the contact between relevant spikes and the core that the neutralization signal preventing secondary uncoating is made. If one assumes that the ratio of relevant to irrelevant spikes is 1 to 70, then the binding of one IgG is capable of causing neutralization by binding to a relevant spike (i.e. single hit kinetics) but on average 70 IgG molecules have to bind before a relevant spike is hit. Interestingly a very similar mechanism of neutralization has been proposed for poliovirus where again the kinetics and stoichiometric data appear at odds [17]. There are four IgG molecules bound per poliovirus particle at 63% neutralization. An alternative stepwise model of neutralization was also suggested [17], where the binding of each IgG reduced the infectivity of each individual poliovirus particle by a factor of 3/4. Unfortunately the data were not sufficiently precise to distinguish between this and the critical site model.

MORE THAN ONE MECHANISM OF IgG-NEUTRALIZATION MAY BE OPERATING AT THE SAME TIME

We have argued that IgG can neutralize influenza virus by binding to the haemagglutinin spike inducing an irreversible conformational change in the subviral core which prevents secondary uncoating of the virus. If this is the case, what role do aggregation of virus and inhibition of virus attachment, which occur at lower IgG concentrations, play in neutralization?

When low IgG concentrations were used then good agreement was found (with the monoclonal antibody HC61 (Fig. 5), but less so with other neutralizing IgG2a monoclones) between the potential loss of infectivity due to the combined effects of aggregation and inhibition of attachment, and the actual loss of infectivity found. This could be taken as implying a causal relationship between these phenomena and neutralization.

If the binding of a single IgG molecule to the virus resulted in neutralization, then it could be argued that only virus already neutralized was aggregated and failed to attach. The quantitative data discussed above show that this is not the case. On average many IgG molecules bind to the virus particle before neutralization occurs but the binding of one IgG was *capable* of causing

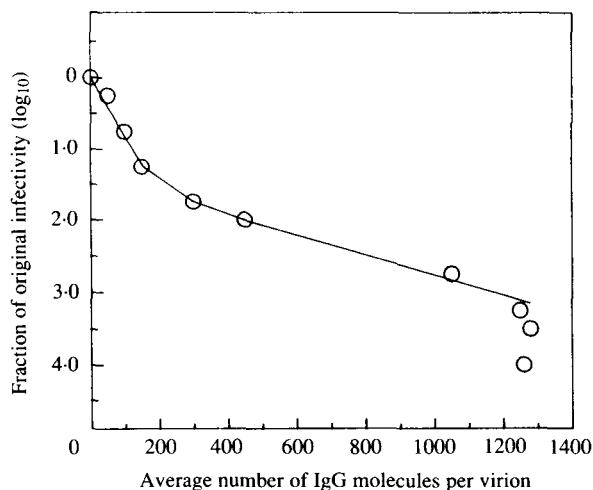


Fig. 8. Comparison of the amount of neutralization of FPV/R by a monoclonal IgG2a (HC10) with the number of ^{125}I -labelled IgG molecules bound per virus particle. From reference 8.

neutralization. The neutralization-relevant spike hypothesis resolves this dilemma but it is unlikely that either aggregation or inhibition of virus attachment are dependent on whether IgG binds to neutralization-relevant spikes or not. The proportion of relevant to irrelevant spikes will be constant within a given virus preparation and therefore the probability of an IgG molecule binding to a relevant spike will also be constant. If the critical site model of influenza virus neutralization only was operating then a straight line should result when neutralization is plotted against the number of IgG molecules bound. Fig. 8 shows that this is not the case. At low IgG concentration, when few IgG molecules (< 200 per virion) are bound, neutralization was more efficient than when higher IgG concentrations were used and more IgG molecules (> 200 per virion) are bound. The difference in neutralization efficiency can be explained by introducing mechanisms of neutralization other than the neutralization-relevant spike hypothesis into the process. The initial efficient phase of neutralization shown in Fig. 8 coincides with the conditions in which both aggregation and inhibition of virus attachment to cells occur. It is likely that aggregation and inhibition of attachment either separately or in combination are responsible for the increased neutralization efficiency at low IgG concentration.

DISCUSSION

We believe that what we have learned about the neutralization of influenza A viruses is relevant to all other types of viruses and this leads us to suggest that there are as many mechanisms of neutralization as there are events in the initial stages of infection. However for any permutation of components in the neutralization reaction one particular mechanism would appear to operate, and this is decided by the unique characteristics of the particle itself, the epitope

specificity and isotype of the immunoglobulin, the ratio of immunoglobulin molecules to virus particles and finally the cell itself and its receptors.

The virus occupies a central position. The diversity of virus structures and infectious routes means that the findings pertaining to one type of virus cannot be automatically interpreted as applying to others. It is perhaps not surprising that there is no single mechanism of neutralization operating for all viruses.

The neutralizing antibody molecule is important, both in terms of its epitope specificity and isotype. Even before contact with a cell, virus can be aggregated by antibody with the result that the number of infectious units is reduced. The valency, affinity, ratio of antibody to virus and concentration of reactants will decide on the efficiency of aggregation. There is evidence that aggregation is a mechanism whereby poliovirus is neutralized by IgG [34, 35] but this is entirely distinct from neutralization which results from conformational changes in the virion coat proteins [36]. However with influenza virus there is aggregation only in a narrow range of antibody (IgG, IgA or IgM) to virus ratio and at best it would seem to play only a minor role in neutralization.

Irrespective of isotype, if an antibody binds directly to the attachment site on a viral coat protein it can prevent attachment. If antibody binds nearby the inhibition of attachment will depend upon its molecular dimensions and other properties such as rotational radius. The larger polymeric immunoglobulins IgM and IgA might be intuitively expected to be more efficient 'blockers' than IgG; this appears to be the case with influenza virus but there are few other data to draw on.

With respect to IgG, there is now a considerable body of published work to show that only in a minority of instances of IgG-neutralization (e.g. reovirus, rotavirus, rhinovirus and foot-and-mouth disease virus [37–40]) does inhibition of virus attachment fully account for the loss of infectivity observed under the particular conditions studied. Attachment of other viruses may be reduced by the binding of antibody but the magnitude of the reduction in attachment makes only a contribution to the neutralization found. A very clear illustration of the fact that IgG does not necessarily affect attachment is provided by those neutralization escape mutants which still bind their selecting monoclonal antibody [35, 41]. Secondly viruses have many attachment sites for cells (3000 in influenza virus, 60 in poliovirus) and since the majority of infectivity is neutralized when there are 70 (influenza virus; [8]) or 4 (poliovirus; [17]) molecules of IgG per particle, it seems unlikely that binding of low amounts of antibody would affect attachment.

The concept of neutralization sites is fundamental to the understanding of neutralization. To neutralize influenza virus IgG must bind to one of 4 or 5 antigenic sites on the HA [18]; those that bind to epitopes outside these sites do not neutralize the virus [42–44]. Such non-neutralizing antibodies may be active *in vivo* against virus or virus-infected cells by fixing complement enhancing phagocytosis or mediating lysis of infected cells by antibody-dependent cell cytotoxicity (ADCC); these mechanisms may well be important in protection and recovery from virus infections but are beyond the scope of this article.

Our data with influenza virus underline the importance of the antibody to virus ratio in relation to neutralization. Antibody–virus–cell interactions are affected in profoundly different ways depending on whether or not the conditions are

saturating, sub-saturating or sub-neutralizing. Many studies have used a single concentration of antibody, so that comparisons with other studies which used different antibody concentrations have been confusing.

If neutralized virus attaches to a cell and is internalized normally then neutralization must occur intracellularly. Some enveloped viruses such as influenza enter cells by adsorptive endocytosis and then the viral envelope and endosomal membranes fuse to release subviral cores into the cell proper [11, 12]. This fusion is mediated by surface proteins which undergo a conformational alteration induced by the acidification of the endosome compartment [45]. It is therefore conceivable that the bound antibody prevents the conformational alteration and hence fusion, and thereby traps the virus in the endosome. This fuses with primary lysosomes and the virus particle is degraded. Such a mechanism has been described for West Nile virus [46]. Other viruses, including paramyxoviruses and HIV [13] fuse at neutral pH with the plasma membrane but the principle of antibody interfering with fusion remains the same. Evidence with influenza virus is conflicting: although some workers have suggested that neutralizing antibodies prevent conformational change in the HA and block low pH-induced fusion of the virus with both liposomes and erythrocytes [47, 48], others [49] have shown that bound antibody does not affect the acid-induced change in the HA; the issue remains unresolved. It may also be important that the binding of virus to liposomes used to demonstrate fusion in these studies was not mediated by the natural receptor molecule (sialic acid) but through electrostatic attraction and therefore may be giving misleading results.

Further we have suggested above that the erythrocyte which is often used to study fusion events has short receptor molecules which are not typical of the majority of cell types and would not therefore appear to be the most appropriate cell for the study of neutralization. The way in which a cell type reacts with neutralized virus has received little attention. The erythrocyte is one example but other cells which show quantitative or qualitative differences in neutralization flag this as a factor meriting closer attention [50–52]. For example, one monoclonal IgG neutralized La Crosse virus on BHK cells but not on a mosquito cell line while a second monoclonal IgG to the same protein neutralized on mosquito but not BHK cells [53]. It seems likely that properties of the cell receptor unit used by the virus to attach to the cell membranes are crucial here. Such molecules, now being identified, are normal components of the cell surface. Not surprisingly they are a diverse collection of molecules, playing various roles in homeostatic and immune recognition processes [9].

Data outlined above suggest that IgG neutralization of influenza virus occurs at a stage following endosomal fusion. This would imply that a signal, presumably conformational, is transmitted from the surface protein to which the antibody binds to the subviral core. Such an event is a common means of communication across membranes for homeostatic mechanisms, intercellular communication and antigen recognition whereby ligands bind to receptors on the outside of the cell and trigger intracellular processes. Antibodies can mimic the effect of some ligands, for example in stimulating lymphocyte activation or hormone receptors. An analogous situation may occur with enveloped viruses although none has been conclusively demonstrated to date. However, antibodies are known to induce

conformational changes in virion proteins [54,55]. We believe that IgG-neutralization of influenza virus blocks a late stage of infection and prevents uncoating of the subviral particle (secondary uncoating) [28] but with other systems it is possible that viral enzymes within the virus particle are inhibited.

ACKNOWLEDGEMENTS

We are pleased to acknowledge support from the Science and Engineering Research Council, the Wellcome Trust and the Ministry of Defence. Fig. 1 was adapted from ref. 56, Figs. 3, 4 and 5 from ref. 16 and Fig. 8 from ref. 8, all with permission.

M. C. OUTLAW AND N. J. DIMMOCK,
*Department of Biological Sciences,
University of Warwick,
Coventry CV4 7AL*

REFERENCES

1. Dimmock NJ. Mechanisms of neutralization of animal viruses. *J Gen Virol* 1984; **65**: 1015–22.
2. Dimmock NJ. Multiple mechanisms of neutralization of animal viruses. *TIBS* 1987; **12**: 70–5.
3. Iorio RM. Mechanisms of neutralization of animal viruses: monoclonal antibodies provide a new perspective. *Microbiol Path* 1988; **5**: 1–7.
4. Svehag S-E. Formation and dissociation of virus-antibody complexes with special reference to the neutralization process. *Prog Med Virol* 1968; **10**: 1–63.
5. Mandel B. Neutralization of animal viruses. In: *Advances in virus research*, volume 23. New York: Academic Press, 1978: 205.
6. Mandel B. Interaction of viruses with neutralizing antibodies. In: Fraenkel-Conrat H and Wagner RR, eds. *Virus-host interactions. Comprehensive virology*, volume 15. New York: Plenum Press, 1979: 37.
7. Mandel B. Virus neutralization. In: van Regenmortel MHV and Neurath AR, eds. *Immunochemistry of viruses*. Amsterdam: Elsevier Science Publishers, 1985: 53–70.
8. Taylor HP, Armstrong SJ, Dimmock NJ. Quantitative relationships between an influenza virus and neutralizing antibody. *Virol* 1987; **159**: 288–98.
9. Lentz TL. The recognition event between virus and host cell receptor: a target for antiviral agents. *J Gen Virol* 1990; **71**: 751–66.
10. Matlin KS, Reggio H, Helenius A, Simons K. Infectious entry pathway of influenza virus in a canine kidney cell line. *J Cell Biol* 1981; **91**: 601–13.
11. Marsh M. The entry of enveloped viruses into cells by endocytosis. *Biochem J* 1984; **218**: 1–10.
12. Patterson S, Oxford JS. Early interactions between animal viruses and the host cell: relevance to viral vaccines. *Vaccine* 1986; **4**: 79–90.
13. McClure MO, Marsh M, Weiss RA. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. *EMBO J* 1988; **7**: 513–18.
14. Russell PH. Newcastle disease virus and two influenza viruses: differing effects of acid and temperature on the uptake of infectious virus into bovine and canine kidney cell lines. *Arch Virol* 1986; **88**: 159–66.
15. Outlaw MC, Dimmock NJ. Mechanisms of neutralization of influenza virus on mouse tracheal epithelial cells by mouse monoclonal polymeric IgA and polyclonal IgM directed against the viral haemagglutinin. *J Gen Virol* 1990; **71**: 69–76.
16. Outlaw MC, Armstrong SJ, Dimmock NJ. Mechanisms of neutralization of influenza virus in tracheal epithelial and BHK cells vary according to IgG concentration. *Virology* 1990; **178**: 478–85.

17. Icenogle J, Shiwen H, Duke G, Gilbert S, Rueckert RR, Anderegg J. Neutralization of poliovirus by a monoclonal antibody: kinetics and stoichiometry. *Virology* 1983; **127**: 412–25.
18. Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of the Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature (London)* 1981; **289**: 373–8.
19. Taylor HP, Dimmock NJ. Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. *J Exp Med* 1985; **161**: 198–209.
20. Taylor HP. The interaction of influenza virus with neutralizing antibody [PhD thesis]. University of Warwick, 1986.
21. Eisenlohr LC, Gerhard W, Hackett CJ. Role of receptor-binding activity of the viral haemagglutinin molecule in the presentation of influenza virus antigens to helper T cells. *J Virol* 1987; **61**: 1375–83.
22. Jackson RL, Segrest JP, Kahane I, Marchesi VT. Studies on the major sialoglycoprotein of the human red cell membrane. *Biochem* 1973; **12**: 3131–8.
23. Viitala J, Jarnefelt J. The red cell surface revisited. *TIBS* 1985; **10**: 392–5.
24. Burton RD. Is IgM-like dislocation a common feature of antibody function? *Immunol Today* 1986; **7**: 165–7.
25. Feinstein A, Richardson N, Taussig MJ. Immunoglobulin flexibility in complement activation. *Immunol Today* 1986; **7**: 169–74.
26. Pumphrey R. Computer models of human immunoglobulins. Shape and segmental flexibility. *Immunol Today* 1986; **7**: 174–8.
27. Possee RD, Schild GG, Dimmock NJ. Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus *in vivo* by inhibiting virion transcriptase activity. *J Gen Virol* 1982; **58**: 373–86.
28. Rigg RJ, Carver AS, Dimmock NJ. IgG-neutralized influenza virus undergoes primary, but not secondary uncoating *in vivo*. *J Gen Virol* 1989; **70**: 2097–109.
29. Lafferty KJ. The interaction between virus and antibody. I. Kinetic studies. *Virology* 1963; **21**: 61–75.
30. Della-Porta AJ, Westaway EG. A multi-hit model for the neutralization of animal viruses. *J Gen Virol* 1977; **38**: 1–19.
31. Peiris JSM, Porterfield JS, Roehrig JT. Monoclonal antibodies against the flavivirus West Nile. *J Gen Virol* 1982; **58**: 283–9.
32. Chanas AC, Gould EA, Clegg JCS, Varma MGR. Monoclonal antibodies to Sindbis virus glycoprotein E1 can neutralize, enhance infectivity and independently inhibit haemagglutination or haemolysis. *J Gen Virol* 1982; **58**: 37–46.
33. Armstrong SJ, Outlaw MC, Dimmock NJ. Morphological studies of the neutralization of influenza virus by IgM. *J Gen Virol* 1990; **71**: 2313–19.
34. Thomas AAM, Brioen P, Boeye A. A monoclonal antibody that neutralizes poliovirus by crosslinking virions. *J Virol* 1985; **54**: 7–13.
35. Thomas AAM, Vrijnsen R, Boeye A. Relationship between poliovirus neutralization and aggregation. *J Virol* 1986; **59**: 479–85.
36. Mandel B. Neutralization of poliovirus: a hypothesis to explain the mechanism and one-hit character of the neutralization reaction. *Virology* 1976; **69**: 500–10.
37. Lee PWK, Hayes EC, Joklik WK. Protein $\sigma 1$ is the reovirus cell attachment protein. *Virology* 1981; **108**: 156–63.
38. Kukuvara N, Yoshie O, Kitaoka S, Konno T. Role of VP3 in human rotavirus internalization after target attachment via VP7. *J Virol* 1988; **62**: 2209–18.
39. Colonna RJ, Callahan PL, Leippe DM, Rueckert RR, Tomassini JE. Inhibition of rhinovirus attachment by neutralizing monoclonal antibodies and their Fab fragments. *J Virol* 1989; **63**: 36–42.
40. Fox G, Parry NR, Barnett PV, McGinn B, Rowlands DJ, Brown F. The cell attachment site of foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). *J Gen Virol* 1989; **70**: 625–37.
41. McCahon D, Crowther JR, Belsham GJ, et al. Evidence for at least four antigenic sites on type O foot-and-mouth disease virus involved in neutralization: identification by single and multiple site monoclonal antibody-resistant mutants. *J Gen Virol* 1989; **70**: 639–45.
42. Breschkin AM, Ahern J, White DO. Antigenic determinants of influenza virus haemag-

- glutinin. VIII. Topography of the antigenic regions of influenza virus determined by competitive radioimmunoassay with monoclonal antibodies. *Virology* 1981; **113**: 130–40.
43. McLain L, Dimmock NJ. Protection of mice from lethal influenza by adoptive transfer of non-neutralizing haemagglutinin-inhibiting IgG obtained from the lungs of infected animals treated with defective interfering virus. *J Gen Virol* 1989; **70**: 2615–24.
 44. Gitelman AK, Kaverin NV, Kharitonov IG, Rudneva IA, Skylanskaya EL, Zhadanov VM. Dissociation of the haemagglutinin inhibition and the infectivity neutralization in the reactions of influenza A/USSR/90/70 (H1N1) virus variants with monoclonal antibodies. *J Gen Virol* 1988; **67**: 2247–51.
 45. Wharton SA. The role of influenza virus haemagglutinin in membrane fusion. *Microbiol Sci* 1987; **4**: 119–24.
 46. Gollins SW, Porterfield JS. A new mechanism of the neutralization of enveloped viruses by antiviral antibody. *Nature (London)* 1986; **321**: 244–6.
 47. Kida H, Yoden S, Kuwabara M, Yanagawa R. Interference with a conformational change in the haemagglutinin molecule of influenza virus by antibodies as a possible neutralization mechanism. *Vaccine* 1985; **3** (suppl): 219–22.
 48. Yoden S, Kida H, Kuwabara M, Yanagawa R, Webster RG. Spin-labelling of influenza virus haemagglutinin permits analysis of the conformational change at low pH and its inhibition by antibody. *Virus Research* 1986; **4**: 251–61.
 49. Webster RG, Brown LE, Jackson DC. Changes in the antigenicity of the haemagglutinin molecule of H3 influenza virus at acidic pH. *Virology* 1983; **126**: 587–99.
 50. Kjellen L, von Zeipel G. Influence of host cells on the infectivity and neutralizability of echovirus type 4 (Pesacek). *Intervirology* 1984; **22**: 32–40.
 51. Kjellen L. A hypothesis accounting for the effect of the host cell on neutralization-resistant virus. *J Gen Virol* 1985; **66**: 2279–83.
 52. Kennedy-Stoskopf S, Narayan O. Neutralizing antibodies to visna lentivirus: mechanism of action and possible role in virus persistence. *J Virol* 1986; **59**: 37–44.
 53. Grady LJ, Kinch W. Two monoclonal antibodies directed against La Crosse virus show host-dependent neutralizing activity. *J Gen Virol* 1985; **6**: 2773–6.
 54. Lubeck M, Gerhard W. Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecule are induced by the binding of monoclonal antibodies. *Virology* 1982; **118**: 1–7.
 55. Colman PM, Laver WG, Varghese JN, et al. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature (London)* 1987; **326**: 358–63.
 56. Lamb RA. The influenza virus RNA segments and their encoded proteins. In: Palese P, Kingsbury DW, eds. *Genetics of influenza viruses*. New York: Springer-Verlag, 1983.