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Brief Review

Neutralization of animal virus infectivity by antibody

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Summary

Neutralization is the ability of antibody to bind to and inactivate virus infectivity under defined conditions *in vitro*. Most neutralizing antibodies also protect animals *in vivo*, but protection is more complex as it also involves interaction of antibody with cells and molecules of the innate immune system. Neutralization by antibody can be mediated by a number of different mechanisms: by aggregation of virions, destabilization of the virion structure, inhibition of virion attachment to target cells, inhibition of the fusion of the virion lipid membrane with the membrane of the host cell, inhibition of the entry of the genome of non-enveloped viruses into the cell cytoplasm, inhibition of a function of the virion core through a signal transduced by an antibody, transcytosing IgA, and binding to nascent virions to block their budding or release from the cell surface. The mechanism of neutralization is determined by the properties of both a virion epitope and the antibody that reacts with it. Further, since a virus has at least several unique epitopes sited in different locations on the virion, and since the paratope and other properties of the reacting antibody can vary, this means that a virus can be neutralized by several different mechanisms. Understanding the processes of neutralization informs the creation of modern vaccines, and gives valuable insights into virus-cell interactions.

Introduction

Antibody plays a pivotal role in the control of many viral infections and is mainly responsible for the prevention of re-infection in convalescent or immunised hosts. A major goal of vaccine developers is the elicitation of potent antibodies that prevent the morbidity and mortality caused by key viral pathogens. However, while in the past this has largely been achieved empirically, modern vaccine development is seeking an understanding of the mechanisms by which antibodies bring about protection in order to make better products. Protection by antibody *in vivo* is complex and difficult to study, so neutralization – the study of the ability of antibody to bind to virions and inactivate virus infectivity under carefully defined conditions *in vitro* is an important half-way house. For simplicity, we will deal here with the interaction of viruses and their specific antibodies only, and will not dwell on the interaction of virus-antibody complexes with important immune system components such as complement, and their role in antibody-mediated cellular cytotoxicity.

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Table 1. An estimate of the different mechanisms used by antibodies to neutralize virus infectivity

Mechanism of neutralization*	Approximate number of times the mechanism has been recorded	Some examples of viruses which exemplify the mechanism	References
(a)	+	Poliovirus	[4, 49, 50]
(b)	±	Poliovirus, foot and mouth disease virus	[5, 9, 30]
(c)	++	Newcastle disease virus, human rhinovirus, HIV-1	[24, 36, 43]
(d)	+++	Hendra virus, Nipah virus, influenza A virus, HIV-1	[3, 15, 21, 48, 58]
(e)	±	HIV-1	[35]
(f)	+	Influenza A virus, HIV-1	[31, 37]
(g)	±	Influenza A virus	[28, 29]
(h)	+	Influenza A virus	[52, 57]

* See Fig. 1.

As detailed below, the means by which antibody mediates neutralization can also be complex, and there appears to be no single mechanism of neutralization that describes all situations, despite several heroic attempts to formulate a unifying model. The topic of virus neutralization has a long history, but still remains contentious [7, 13, 14, 27]. Rather than attempting to generalize, we prefer to approach neutralization by defining each virus-antibody interaction and the conditions affecting it (Fig. 1) and the frequency with which each neutralization process is found (Table 1). What is clear is that any one virus can be neutralized in several different ways that are determined primarily by the specificity of the reacting antibody. For this reason, analysis is only possible using monoclonal antibodies. In this way, individual neutralization processes can be understood, and hopefully useful generalizations will eventually arise. What follows is an overview of the mechanisms of antibody-mediated neutralization. It is beyond the scope of this review to present a comprehensive account, and readers are urged to consult current literature in order to gain a more complete appreciation of this intriguing subject and the various models that have been put forward to explain the neutralization process.

Mechanisms of virus neutralization

Aggregation of virus particles

Upon successfully entering a permissive cell, each infectious virion is capable of replicating itself. Aggregation reduces the number of virions able to initiate an independent infectious event, and reduces overall infectivity [4, 49, 50]. This reduction is directly proportional to the number of particles in the aggregate so that, for example, an aggregate of five virions results in a five-fold drop in infectivity (Fig. 1a). However individual virions within the aggregate are infectious and disaggregation can restore infectivity. The number of antibody-binding sites ranges from two on a monomeric antibody molecule (Fig. 2) to ten on a pentameric IgM molecule. About half of all antibodies tested are obligate bivalent binders, meaning that they are orientated so that both arms of the antibody (FAbs) bind to the same virion and no aggregation can take place. (However, when the antibody concentration is saturating, some FAb arms are forced to point into solution and are available for aggregation.) The other antibodies are obligate monovalent binders, and are orientated with one arm pointing into solution, and have the potential to cause aggregation [20]. Stable aggregation is likely to need ligation by several antibody molecules. The ability of an antibody to bind to a virion bivalently depends on the flexibility of its hinge region, which is determined by the antibody isotype, the span of the FAb arms, and distance apart of the epitopes. Aggregation would presumably contribute to protection from virus infection *in vivo* and, in addition, would facilitate clearance of virions from circulation as these larger particulates are more readily phagocytosed and degraded. Aggregation is demonstrable *in vitro* only within a relatively narrow range of antibody: virus

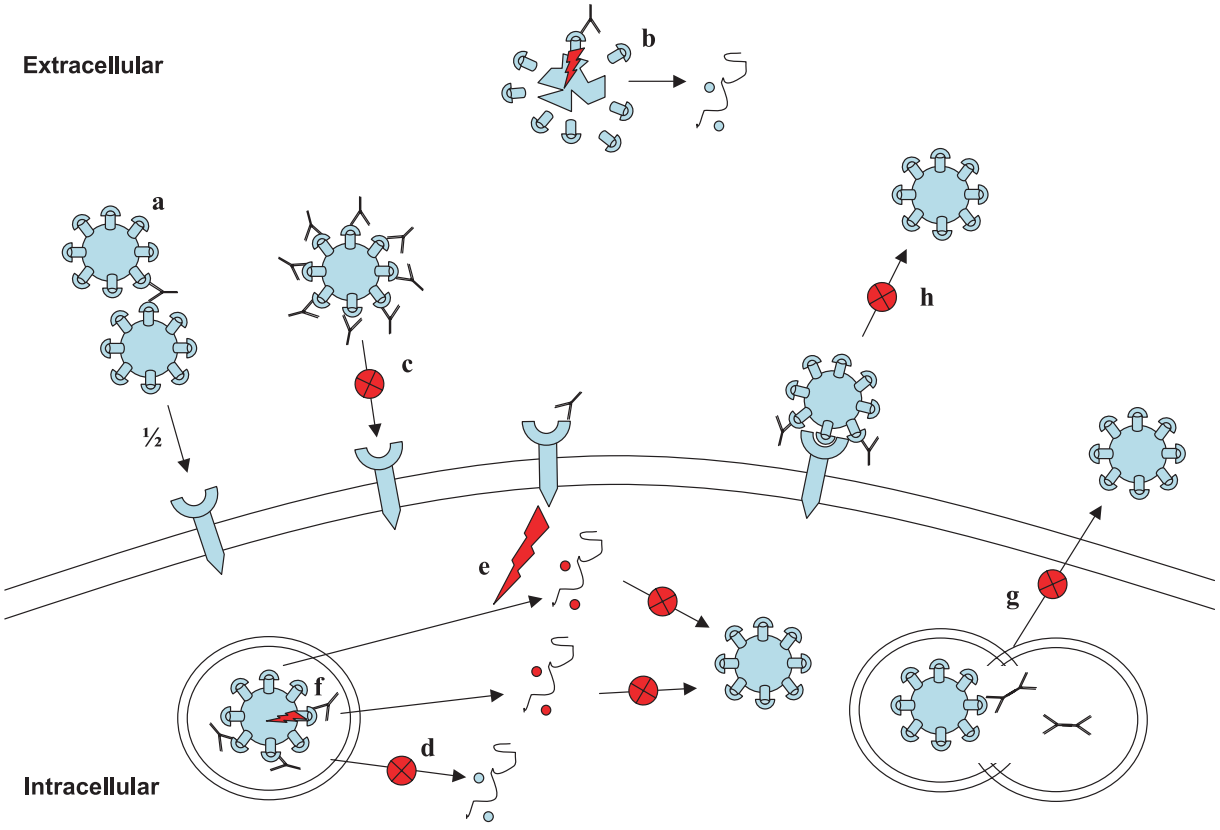
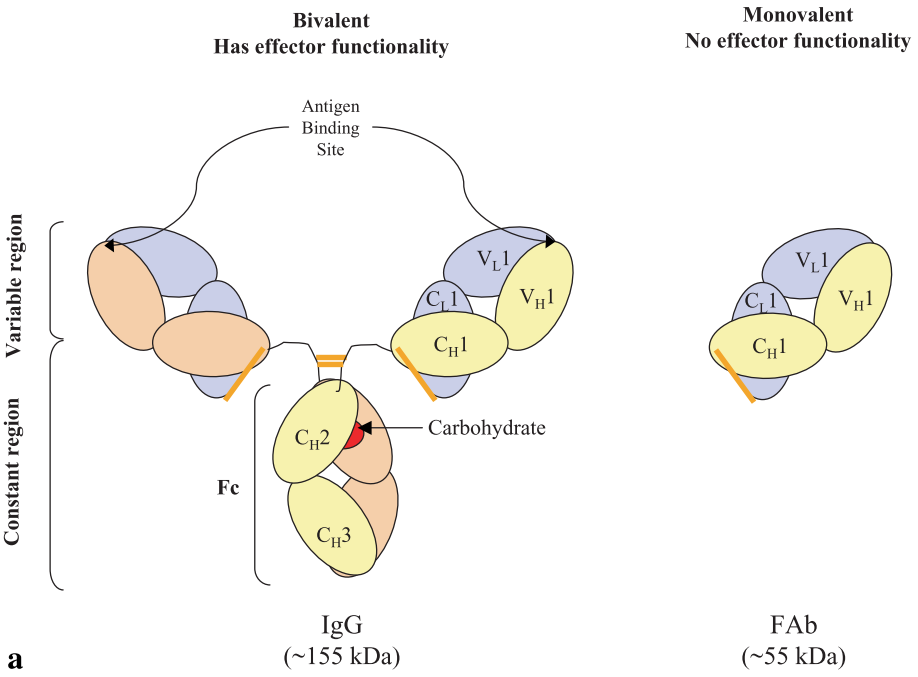


Fig. 1. An overview of the mechanisms through which antibodies neutralize virus infectivity. Details of processes **a**–**h** can be found in the text. Virions are represented as spiked circles, cellular receptors as a blue semicircle mounted on an arrow, IgG as a Y, and blocked events as an arrow carrying an X mounted in a red circle. **a** Antibody aggregates virions and reduces the number of infectious centres. The fraction shown (1/2) represents the loss in infectivity. **b** Antibody that mimics cell receptor ligation binds virions and leads to the disruption of the virion capsid (red lightning) and premature release of the genome. **c** Antibody inhibits virion attachment by blocking receptor engagement. **d** Antibody inhibits fusion/entry occurring inside an endocytotic vesicle (illustrated) or at the cell membrane (not shown). **e** Antibody binds to a cell-surface protein and results in the transduction of a signal into the cell (red lightning) that aborts the infection by modification of the replication complex. **f** Post-entry neutralization by transmission of an allosteric signal via the virus surface protein to the virion core (shown as red lightning). The core is released into the cytoplasm but is defective and unable to replicate. As in (e), this process is poorly understood. **g** Transcytosing IgA antibodies (represented as a double-ended Y) neutralize virus when their respective vesicles fuse. **h** Antibody binds nascent virions and blocks their budding or release from the cell surface

ratios and produces a characteristic neutralization curve which peaks at the point of equivalence. As the antibody concentration increases, aggregation and neutralization decrease.

Destabilization of virion structure

The binding of certain MAbs to poliovirus results in the destabilization of the virion and the release of the viral genome into solution (Fig. 1b). MAb 35-1f4 aggregates poliovirus under physiological conditions, but triggers the release of viral RNA when bound in a low-ionic-strength environment [5]. At physiological ionic strength, the same MAb also destabilises virions at 38 and 39 °C, but not at 37 °C [9]. Some MAbs to foot-and-mouth disease virus also disrupt virion capsids, but do so at 37 °C and normal ionic conditions [30]. It seems likely that these MAbs act by mimicking cell receptors, which normally bind virions and, in the context of a target cell, result in uncoating and infection.



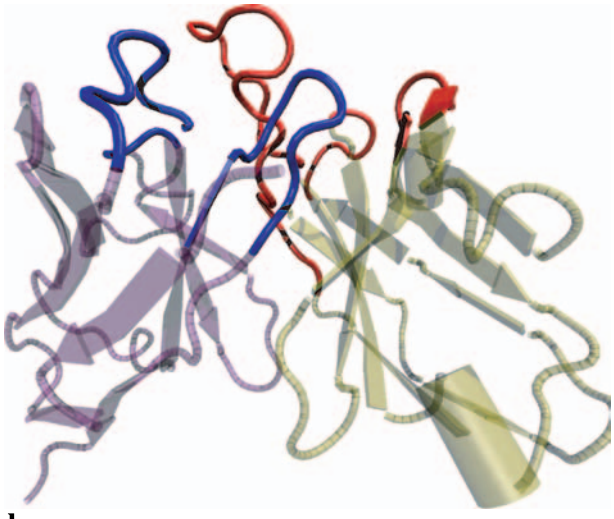


Fig. 2. **a** Schematic representation of an IgG (immunoglobulin G) antibody (left panel) and its FAb fragment (right panel). The IgG is assembled from two identical heavy–light chain pairs shown in lilac (light chains) and yellow or peach (heavy chains), the orange bars represent disulphide bonds which vary in number between isotypes. Each antibody domain comprises approximately 110 amino acids and is represented by a coloured ellipse. The antibody is functionally divided into a variable region that contains the epitope-specific binding site or paratope and a constant region that is less variable, is not epitope-specific and mediates the biological effector functionality. Digestion of IgG with papain yields an Fc fragment from the constant region (not shown) and two monovalent FAb fragments, each of the latter having a single paratope but possessing none of the other IgG effector functions. **b** A ray-traced cartoon showing the antibody paratope produced from the crystal co-ordinates of the neutralizing HIV-specific b12 FAb. The V_L domain is shown in yellow and the V_H domain in lilac. Each domain has three hypervariable loop structures termed complementarity-determining regions (CDRs), represented as blue loops from the V_L domain and red loops from the V_H domain. The CDRs make the principal contacts with the epitope and are the determinants of antibody specificity, the most important of which is usually the third CDR of the heavy chain, which is situated at the centre of the binding site. The image was rendered in the VMD software v1.8.2, and the crystal co-ordinates are available from the protein databank under accession code 1HZH

Inhibition of virion attachment to the target cell

Attachment of the virion to the target cell is the first stage of infection and is mediated by the specific interaction of a viral surface protein(s) with a cell receptor(s). Most commonly, this is a protein–protein interaction, but cell receptors may also be lipid or carbohydrate moieties. Both enveloped and non-enveloped virions attach to cells via specialised binding domains situated on their capsid/membrane-anchored spike proteins with a cognate region on the cell receptor. Virus attachment, and usually infection, is restricted to cells expressing the cognate receptor. Attachment can be specifically inhibited by an antibody that blocks the virion protein engagement site (Fig. 1c). Some cell-receptor-specific antibodies can also inhibit virus attachment and result in neutralization. To block attachment, an antibody does not need to bind directly to the virion protein binding site but is required only to occlude it or interfere with its function in some other way. An example is the neutralization of human rhinovirus type 14 by MAbs that inhibit its attachment to its ICAM-1 cell receptor [43]. The attachment site on the virion surface is situated in a recessed cleft or canyon that is inaccessible to antibody, but is surrounded by an immunogenic ridge. Neutralizing MAbs bind bivalently to this ridge, bridging the canyon, and sterically prevent receptor engagement. Other examples of MAbs that neutralize by inhibiting cell attachment are MAb F105 to HIV-1 [36], and several MAbs to Newcastle disease virus [24].

MAbs are frequently reported to inhibit virus-cell attachment, but often this inhibition is far less than the amount of observed neutralization. In order to establish cause and effect, studies of inhibition of attachment and neutralization need to be conducted under *identical* experimental conditions – and often are not.

There are other antibodies (including the large pentameric IgM) that bind to the virion and do not inhibit attachment even when present in saturating amounts. Such antibodies may neutralize by other mechanisms (see below) or may be non-neutralizing. For example, a rabies virus mutant binds up to 1000 IgG (MAb 30AA5) molecules per virion (which is close to saturation) without being neutralized [16]. Further, evidence showing that there are antibodies that neutralize after virions have attached to the cell (post-attachment neutralization – see below), proves conclusively that there are more ways to effect neutralization than inhibiting virus attachment.

Inhibition of virus attachment requires antibody to sterically blockade receptor engagement, but most virions possess many copies of the viral attachment protein, each of which is potentially capable of binding the virion to its target cell. For instance, influenza A virus presents approximately 800–1000 haemagglutinin spikes on the virion surface. Each spike is a homotrimer, giving a total of up to 3000 attachment sites. With a 1:1 equivalence and monovalent antibody binding, 3000 antibody molecules would be required to block every attachment site, but in reality each MAb has sufficient mass to occlude more than 1 spike. A virion is likely to require multiple contacts with the host cell to stabilise the interaction, but because of the number of attachment sites, multiple antibody molecules need to be bound to a virion to prevent its attachment to the cell. Extending this argument suggests that larger virions with more attachment sites require more bound antibody to block attachment to cells than do smaller virions. This proposal is supported by the finding that for some viruses the number of IgG molecules bound at 63% neutralization is given by the formula $[n = 0.0033 \times \mathrm{A}]$, where n is the number of bound IgG molecules and A is the surface area of the virion [7].

A general assumption is that bound antibodies project radially from a virion. This may not be so, especially with enveloped virions. A single IgG molecule is approximately the same size as a haemagglutinin trimer of influenza virus, and if the IgG were bound perpendicularly to the orientation of the spike it would reside within the fringe of the surface proteins, and hence would not impede engagement of the cell receptor [42]. However the larger antibodies (IgM and dimeric IgA), whatever was their orientation, would protrude beyond even large spikes. As a further rider, these antibodies can also bind in a 'staple' conformation with all the FAb regions at right angles to the rest of the molecule, so that the span of the molecule is shortened.

This may be the reason that some IgA or IgM antibodies did not inhibit the attachment of Dugbe virus [19] or rabies virus [16] even when present at saturating concentrations. An antibody that binds to an epitope that is situated close to the viral membrane will also not protrude much above the surface of the virion even if the antibody is orientated radially. Steric inhibition is maximal when the epitope is situated towards the tip of the virus attachment protein. However the dimensions of the cell receptor are also a factor. If the receptor is significantly shorter than the antibody, antibody may inhibit attachment; if it is longer, and the antibody is not bound directly to the attachment site, the receptor may be able to make contact with the virion and initiate infection.

The cell type itself can influence the extent of neutralization, and even dictate neutralization absolutely. In one study, La Crosse bunyavirus was neutralized by a MAb in BHK (baby hamster kidney) cells but was insensitive to neutralization by another MAb. In a mosquito cell line the situation was reversed [18]. The probable explanation is that the virus utilised a different receptor in each cell type.

Inhibition of virus entry into the target cell

Entry into the target cell poses a fundamentally similar challenge to both enveloped and non-enveloped virions, although aspects of their entry mechanisms differ. Enveloped viruses enter the cell by fusion of virion and cell lipid membranes in a process mediated by the viral surface glycoproteins. Those fusion proteins of viruses that have been extensively studied (influenza virus and HIV-1) are present on the virion in a metastable pre-fusion conformation that is activated by interaction with the host cell so that a hydrophobic fusion peptide is released and inserted into the cell membrane. The fusion protein then undergoes complex structural rearrangements to bring the viral and cellular membranes into close contact and promotes lipid mixing. Low pH (influenza virus) or the binding to cellular receptors (HIV-1) activate the fusion process. At no stage during the fusion/entry process are the internal viral proteins exposed to antibody, and they are not a target for fusion inhibition. Binding of antibody to the surface spike protein may prevent the subsequent interaction of the virion with a cellular fusion receptor in an analogous manner to the inhibition of attachment, or may block the membrane fusion process by impeding the juxtaposition of the viral and cellular membranes (Fig. 1d). Alternatively, bound antibody may stabilise the pre-fusion conformation, and prevent activation of the fusion process. Premature fusion activation may also be mediated by the virion binding an antibody that mimics the normal receptor interaction. Some MAbs are capable of inhibiting virus fusion after attachment (post-attachment neutralization; PAN) and so are unlikely to inhibit fusion by the latter mechanism. Antibodies that effect PAN may not be able to neutralize free virions. MAb SAR1 binds to the gp41 of HIV-1 but may require the virion to bind to receptors to create or expose its epitope in a neutralization-compatible form [21, 39]. This MAb binds to free virions but does not trigger premature fusion activation or compete with the fusion process following virus-cell attachment, and it may be that the MAb is binding to infection-irrelevant gp41 from which gp120 has dissociated. Current models of enveloped virus fusion suggest that more than one spike is required to complete the fusion process. Several spikes are co-operatively recruited into a complex which establishes the fusion pore. If antibody binds to a sufficient number of spikes to prevent the formation of the fusion complex, then the virus will be unable to breach the cell membrane.

Epitope exposure is particularly relevant to PAN. For example, the epitope of MAb 17b, which is specific for the HIV-1 envelope protein component gp120, is partially occluded or unformed on free virions, and the MAb binds and neutralizes poorly. However, binding of gp120 to the CD4 receptor causes conformational changes in gp120 that improve the availability or conformation of the 17b epitope, and increases neutralization [48].

Non-enveloped virions typically enter the host cell by hijacking the endocytic pathway, and so are internalised in a membrane-bound vesicle. Escape (usually of a subviral particle or viral genome) from the vesicle is mediated by capsid protein that forms a channel in the wall of the vesicle. Viruses such as picornaviruses possess a structure that is

similar to certain enveloped virus fusion peptides. Exposure of this lipophilic peptide requires conformational re-arrangement of the capsid protein and may be triggered by changes in ionic strength and low pH within acidified endosomes. Bivalently bound antibody may stabilise the capsid and prevent these structural rearrangements – in contrast to the antibodies, mentioned above, that cause uncoating of the capsid. This mechanism is consistent with single picornavirus virions that bound only small quantities of IgG, but lost infectivity [23] – the implication being that bound MAb prevented the co-operative uncoating of the capsid proteins. Bound antibody may interfere with receptor-mediated endocytosis at the plasma membrane and prevent internalization, or conversely, may target virions into a degradative pathway by engaging with cellular Fc receptors.

Inhibition of post-entry events

Antibodies cannot cross an intact plasma membrane. Antibody that is endocytosed as a virion-antibody complex is still not in the cytoplasm. Such antibody remains within the vesicle/endosome, enters the lysosomal pathway, and is ultimately degraded. Polymeric secretory IgA can be taken up into a vesicle via receptor-mediated endocytosis at the basolateral surface of a polarized cell and transported to the external apical surface by a process called transcytosis. At the apical surface it is released into the external medium by fusion of the vesicle with the cell membrane. IgA applied to the basolateral surface of polarized epithelial cell monolayers has been shown to neutralize the progeny virions of Sendai and influenza viruses, which bud from the apical surface [28, 29]. It has been suggested that the IgA is endocytosed and meets assembling progeny virions or viral proteins within the cell by the convergence of cellular trafficking pathways prior to budding and release (Fig. 1g). However, the possibility of antibody associating with virus at the apical cell surface cannot be ruled out. IgG does not behave or neutralize in this way.

Despite the fact that antibody does not enter the cell cytoplasm, it has been hypothesised that binding of some antibodies to an epitope on the exterior of the virion may result in neutralization via loss of a function of the post-entry viral core structure (Fig. 1f). Transmission or blocking of an allosteric signal to the virion core by bound antibody, or via virus-specific cell receptors cross-linked by antibody, is a plausible explanation for post-entry neutralization, although no exact mechanism has been defined. The binding of certain MAbs to the CD4 cell receptor expressed on T-helper cells and macrophages prevents reverse transcription from fused HIV-1 [35], and this illustrates how antibody bound to a surface protein can mediate the loss of infectivity after entry has occurred (Fig. 1e). Studies using IgG [37, 40] and IgA [1, 46] directed against influenza virus have demonstrated a mechanism of neutralization at a stage of infection subsequent to fusion that appeared to manifest as a block on transcription from the internalised viral RNP. Despite reaching the nucleus, there was no *de novo* viral RNA synthesis. Disruption of the virus-antibody complex with detergent revealed that the transcriptase complex was fully functional when supplied with capped mRNA and template. It was concluded that conformational re-arrangement of the RNP complex required for transcription did not occur in neutralized virions. In an analogous way, MAb ICR39.3b to the gp120 attachment protein of HIV-1 inhibits a post-fusion event (S. J. Armstrong, L. McLain and N. J. Dimmock, unpublished data), possibly via signal transduction through gp120 to the virus core, via the associated gp41 transmembrane anchor region and its long intravirion tail. The gp41 intravirion tail interacts with the underlying p17 matrix protein [56].

The FAb of the HIV-1 gp120-specific antibody, b12, blocks a post-entry event [31], suggesting that receptor cross-linking is not necessarily a requisite of signal transduction. In contrast, the b12 IgG prevents virus entry, suggesting that either bivalent binding or its larger structure is key to this process. The FAb-mediated post-entry inhibition probably takes place at the same time but is irrelevant as the IgG has a 10-fold higher specific activity than its FAb. While gp41 may transduce a signal to the virion core, it is possible that another membrane protein interacts with the antibody-ligated gp120-gp41 complex and carries out this role. Host-cell-

encoded transmembrane proteins (e.g. HLA and various cell adhesion molecules) that are incorporated into HIV-1 virions are possible candidates.

Finally, inhibition of virion assembly at the cell surface or inhibition of the release of progeny virus constitute post-entry neutralization events. MAbs to influenza A virion surface neuraminidase and M2 proteins prevent the release of virions from the infected cell surface and are neutralizing [52, 57] (Fig. 1h). MAb SAR1 to HIV-1 neutralizes progeny virions when added as a late pulse to the neutralization assay after internalization and replication have taken place, and does not neutralize free virions [21, 39].

Some models of virus neutralization

Neutralization by the occupancy of antigenic sites

One theory put forward to unify the concept of antibody-mediated neutralization is the occupancy model [reviewed in 27], which argues that neutralization results from antibody coating the virion surface and inactivating a sufficient number of functional proteins to inhibit virion attachment or entry [10, 26, 33]. A stricter definition of occupancy theory is not concerned with the number of antibodies bound at neutralization but with the volume that they occupy, at the same time acknowledging the contributory effects of antibody orientation and its ability to interfere with neighbouring virion surface proteins. At the present it is difficult to assess the number of epitopes occupied at neutralization, but measuring the steric contribution of bound antibody is even more challenging.

Statistical analysis of the number of antibodies bound per virion has been used as a measure of occupancy required for neutralization. The model predicts that below a minimum threshold, antibody is bound, but neutralization does not result. This is based on the assumption that there is no incremental loss of infectivity as increasing numbers of antibody molecules are bound up to the critical amount required for neutralization. Thus a virion partially coated with antibody can establish an infection provided there are enough functional proteins to interact with the target cell. The number of bound antibody molecules at 63% neutralization is often measured as this represents the average number of virions that would be inactivated when there is a single neutralization event per virion according to Poisson distribution. Some studies of adenovirus [54] and poliovirus [53] have reported an average of only one antibody bound per virion at neutralization, while neutralization of adenovirus [45], poliovirus [23], influenza A virus [47], and rabies virus [16] required multiple antibodies bound per virion at 63% neutralization. The former findings are hard to justify using an occupancy model as a picornavirus virion is significantly larger than an IgG and has multiple cell receptor binding sites. It is, however, not trivial to measure the stoichiometry of antibody binding at neutralization; experiments must be carefully designed, and neutralization and antibody binding must be measured under identical conditions. Furthermore, the assumption of a Poisson distribution is only valid in a homogenous system and assumes all virions are of the same size, do not aggregate, possess the same number of epitopes, and are incubated with monoclonal antibody. Studies using polyclonal sera are thus more artefact prone than those using monoclonal antibodies.

A prediction of occupancy theory is that different densities of the antibody-binding sites on the virion surface or changes in particle size impact on the numbers of antibody molecules required for a given level of neutralization. Hence larger virions or those possessing more available epitopes will require more bound antibodies in order to neutralize them. This simple idea has support from a study by Burton and co-workers that showed an almost linear relationship between virus particle size and amount of antibody required for neutralization [7, 34]. Such a model could also account for the absence of neutralization by inhibition of attachment or fusion with certain MAbs to the influenza virus neuraminidase and M2 proteins if the density of neuraminidase and M2 are assumed to be lower than the occupancy required to interfere with cell receptor binding by the more abundant haemagglutinin [27, 34]. Non-functional or non-virion-encoded proteins may be a target for neutralization provided the protein density is sufficient to allow

bound antibody to interfere with the functional attachment/entry proteins and is exemplified by the neutralization of HIV-1 virions that have incorporated cellular ICAM-1 [41]. Studies with rabies virus showing that neutralization requires more than 200 IgG molecules per virion also support an occupancy model, but an escape mutant that bound more than 1000 IgG molecules (of MAb 30AA5) per virion was still not neutralized [16]. Another study with a different MAb (#1-46-12) is difficult to reconcile with this model [25]. Here, wild-type virus was neutralized with just 20 IgG molecules bound per virion (approximately 10-fold less than some other G-protein-specific MAbs) and no longer bound to cell receptors even though only 3–4% of the available G protein spikes were occupied by MAb. The same MAb failed to neutralize two escape mutants, one of which (R-31) had lost the cognate epitope and did not bind the MAb. The other mutant virus (R-61) retained the cognate epitope but was not neutralized even with more than 300 IgG molecules bound per virion. Allowing pseudotyped virus particles to form by coinfection with a mixture of mutant and wild-type viruses resulted in a sharp loss of the resistant phenotype of R-61, even with only small amounts of wild-type G protein present. However, incorporation of wild-type G protein into the R-31 mutant (which before phenotypic mixing did not bind IgG) did not significantly alter its sensitivity to the MAb. The authors proposed a ''domino model'' to account for these findings which proposes (a) that the binding of the neutralizing MAb to wild-type G protein induces a conformational shift in G protein spikes that is incompatible with its binding to the cell receptor, and (b) that this change is transmitted to neighbouring spikes. For the mixed-phenotype virions, it was suggested that the R-61 G protein is sensitive to the conformational shift, whereas the R-31 G protein is not.

Neutralization by ligation to a critical binding site

Another take on neutralization is the ''critical binding site'' concept. This theory proposes that antibody binding alone and coating of the virion are not the sole requisites for neutralization, but that certain binding sites are inherently compatible with neutralization whilst others are not [13]. The critical site model is compatible with both single- or multi-hit theories of neutralization. In the case of single-hit neutralization all of the available epitopes are critical to the infectious process. However, this is not a straightforward concept, as a single antibody bound to a virion must act in such a way as to incapacitate all other attachment/entry proteins and/or prevent crucial post-entry processes. For some picornaviruses, the antibody may stabilise the virion structure to prevent uncoating and release of the genome (see above '*Inhibition of virus entry into the target cell*'), while antibody bound to enveloped viruses such as influenza A virus might effect transduction of a signal to the particle core that prevents virus replication (see above '*Inhibition of post-entry events*', and below). For multi-hit phenomena, only certain epitopes are compatible with neutralization, and on average more than one antibody molecule must bind for there to be a statistical chance of it knocking out the critical site. The question then arises that if all epitopes are identical, how can one epitope be a critical binding site while another is not? One possibility is that not all surface proteins are functional but can still bind the MAb. HIV-1, for example, progressively sheds gp120, leaving behind the membrane-anchored gp41. Thus a MAb can bind the same epitope on a gp120-gp41 complex as on gp41 alone, but only the former may be neutralizing. Alternatively, multi-hit neutralization may be accounted for by only some viral surface protein molecules associating with neighbouring molecules or with virion-internal proteins, with neutralization being mediated only when an antibody binds to a complexed protein. Indeed HIV-1 gp41 external protein and the P17 matrix internal protein have been reported to interact [56], and a model has been proposed in which each of the three gp41 C-terminal tails of an intact gp41 trimer insert into a triangular-shaped facet formed in the matrix shell [38]. Conceivably, this could relay an allosteric signal initiated by the gp41-specific antibody to the virion core, or via the matrix to neighbouring spikes, thus inactivating the unligated envelope proteins. Surface spikes not associated with the matrix, e.g. unpaired monomers or dimers,

may be unable to transduce such signals. The concept of signal transduction through a critical binding site thus provides a model for post-entry neutralization mechanisms where the antibody remains external to the cell but affects secondary uncoating or replication [1, 35, 37, 40].

Some MAbs bind to virions but do not neutralize at any concentration, so how does the model account for these? For example, rabies virus can bind 1000 molecules per virion of some MAbs and still not be neutralized [16]. In this case, an explanation consistent with the critical binding site model is that none of the epitopes is a neutralization-critical site i.e. this epitope-paratope pair defines a non-neutralizing interaction. The existence of high affinity, non-neutralizing MAbs is important as it specifies that neutralization requires more than just binding of antibody to functional virion surface spikes [6, 8, 22].

Other insights into neutralizing antibodies

Factors affecting neutralization are many and varied [32], and those concerning the specific mechanisms of neutralization have been discussed above. Some other properties of neutralizing antibodies are selected here for discussion.

Epitope–paratope interactions

A neutralizing antibody must bind specifically to a neutralization-compatible epitope. This prerequisite is met when the epitope and paratope have a complementary charge and conformation. Antibodies that bind small molecules and peptides usually accommodate them in a groove in the paratope structure for high-affinity interactions. However, antibodies that bind linear epitopes on a protein surface tend to have corresponding planar paratopes. A number of antiviral MAbs that recognise discontinuous epitopes that are hidden in recesses on the virion surface have finger-like projections in the paratope that permit close contact to be made (see Fig. 2b). Striking examples are some potent, broad-range human HIV-1-neutralizing antibodies that possess a paratope with a long heavy-chain third complementarity-determining region or loop (CDR-H3). These long loops are commonly found in autoreactive antibodies that are eliminated during the course of immune system maturation, and this may explain why HIV-1 antibodies are uncommon and difficult to elicit.

Binding affinity and kinetics

Two useful measures of epitope-paratope interactions are their affinity (K_D, M), and their rates of association (k_a, $M^{-1}\ s^{-1}$) and dissociation (k_d, s^{-1}). These factors are related by the formula $K_D = k_d/k_a$. Affinity measures the binding strength of one epitope-paratope pair, but when more than one epitope-paratope interaction is considered, then it is more accurate to refer to functional affinity or avidity. Most studies show that affinity probably does not have a profound effect on the level of neutralization but may be a determinant in target range for some viruses. This is illustrated by an engineered anti-HIV-1 gp120-specific FAb b12 that had increased affinity but gave only a modest increase in neutralization of the homologous virus strain [2]. However, the FAb was now able to neutralize previously insensitive heterologous viruses, probably because its affinity now exceeded the minimum threshold for binding. A recent study using MAbs to the gp120 envelope protein of neutralization-sensitive and neutralization-resistant variants of simian immunodeficiency virus (SIV) showed that neutralization-insensitive virus bound MAbs with higher association rate constants than did neutralization-sensitive virus, but these MAbs also had higher dissociation rate constants [44]. This suggested that the interaction was unstable and provided an explanation for the neutralization-resistant phenotype. Affinity did not correlate with neutralization for the MAbs tested.

Concluding remarks

There is evidence from adoptive transfer experiments that many neutralizing antibodies also protect animals *in vivo* [11, 17, 51, 54], but the correlation between *in vitro* neutralization and *in vivo* protection is far from universal. This may be due to differences in the properties of target cells

in vitro and *in vivo*, but also to the biological functions that are mediated by the Fc portion of antibody. There is thus an urgent need to design neutralization test systems that are relevant to the *in vivo* infection. In terms of vaccines, the more we understand about how antibody neutralizes viral infectivity, the better equipped we shall be to devise immunogens that elicit the specificity and isotype of antibody that confers the maximum neutralizing, and eventually, protecting activity. At present there are serious problems in formulating universal principles of neutralization, and it is important to entertain the possibility that no commonality applies to virus-antibody neutralizing interactions.

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