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REVIEW ARTICLE

Occupancy and mechanism in antibody-mediated neutralization of animal viruses

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Neutralization of virus infectivity by antibodies is an important component of immunity to several virus infections. Here, the immunochemical basis for the action of neutralizing antibodies, and what role their induction of conformational changes in the antigen might play, is reviewed. Theories of the mechanisms by which antibodies neutralize virus infectivity *in vitro* are also presented. The theoretical and empirical foundation of the hypothesis that viruses are neutralized by a single antibody per virion is critically reviewed. The relationship between antibody occupancy on virions and the mechanism of neutralization is explored. Examples of neutralization mediated through antibody interference with virus attachment and entry are discussed and test implications of refined theories of neutralization by antibody coating of virions are formulated.

Introduction

Virus infections have different outcomes: they may be eliminated from the host organism, they may persist latently or at a low level of virus replication or they may continue unabated in the face of host defences. It is plausible that viruses can sometimes be eliminated without the involvement of the immune system (Nowak & Bangham, 1996; Phillips, 1996). Immune clearance might occur either in unison or in conflict with such non-immune mechanisms. The relative contribution of antibody (Ab) and cellular immunity to the elimination of different viruses from the organism probably extends across a broad spectrum. Either kind may be crucial to the clearance of some viruses. Potentially, the interplay between these kinds of immunity could be synergistic or antagonistic, so that their real effects cannot be established in isolation. Nevertheless, a role for Ab-mediated immunity has been substantiated through passive immunization of immunodeficient animals: for example, Ab neutralization is both necessary and sufficient for protection against the rhabdovirus vesicular stomatitis virus (VSV) (Bachmann *et al.*, 1994, 1997). Other cases of the importance *in vivo* of neutralizing antibodies (nAb) are described in a recent review (Parren & Burton, 2001), which also covers the effect of the complement system on virus infectivity and neutralization. The requisite properties of nAbs, their mode of action and degree of binding, as well as other aspects of the neutralization of diverse viruses, have been analysed recently (Burton *et al.*, 2000, 2001; Bizebard *et al.*, 2001; Epa & Colman, 2001; Gerhard, 2001; Hewat & Blaas, 2001; Klasse & Sattentau, 2001; Smith, 2001). Here, we discuss an emerging unifying view of virus neutralization: Ab coating of virions is usually both necessary and sufficient for neutralization and such coating often results in a block of virus attachment to target cells, an effect that is sufficient but not necessary for neutralization.

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Virus neutralization by antibody

The neutralization of viruses is defined here as the abrogation of virus infectivity *in vitro* by the binding of Abs to the virion. Thus, the target antigen (Ag) of nAbs does not have to be of virus origin, as long as it is present on the virion. The definition does not include the block of infection by an Ab that binds to a receptor for the virus on the cell surface. It is reasonable to add a further criterion: that nAbs act before the first major biosynthetic event in the virus replicative cycle has taken place (Dimmock, 1984). Then, it is a matter for experimental investigation whether neutralization can block a step between virus entry (reviewed by Klasse *et al.*, 1998) and

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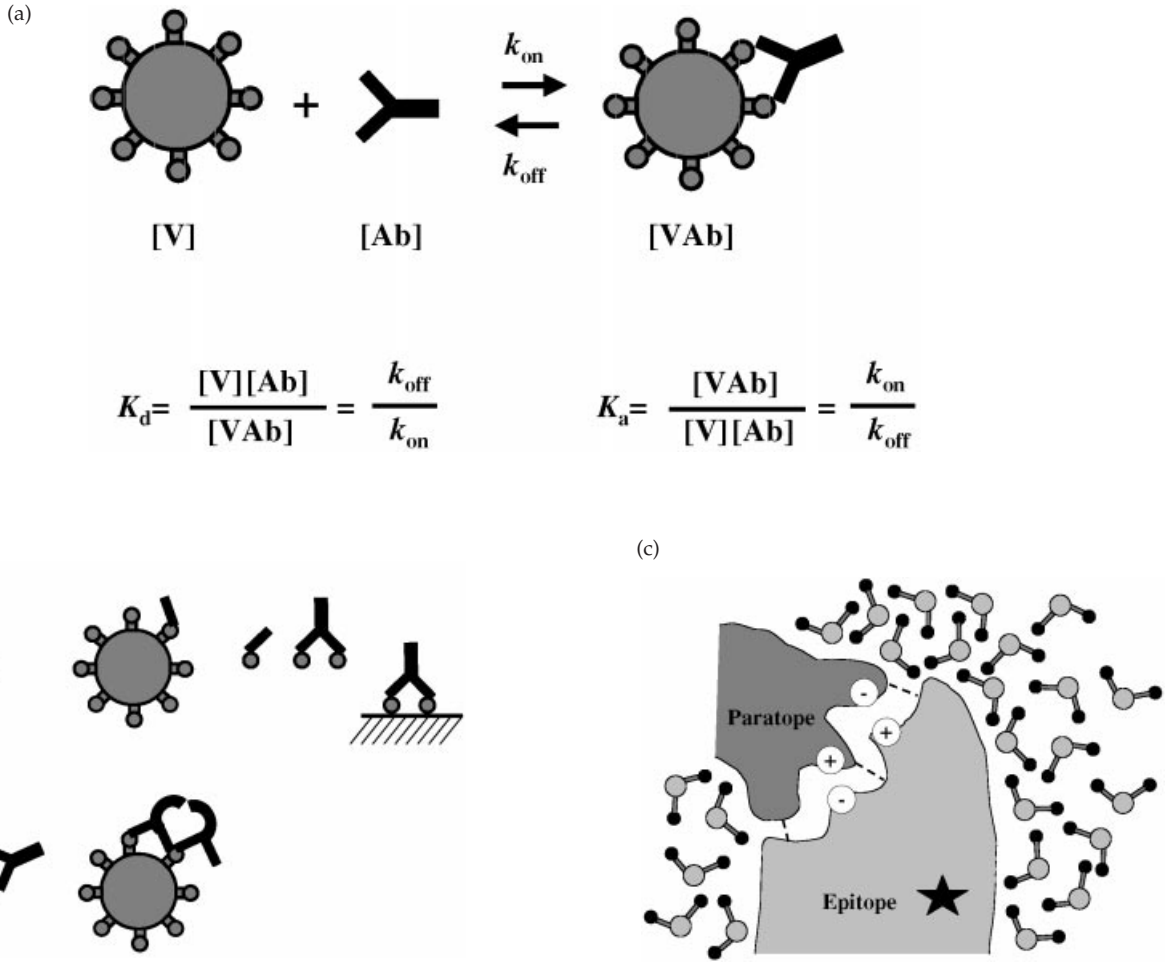

Fig. 1. Ab binding to virions and soluble Ag. (a) The minimum requirement for neutralization is Ab binding to Ags as they are presented on the virion surface. The formation of complexes between Ab and viral Ag can be analysed according to the law of mass action as the reaction approaches equilibrium. The affinity of the Abs for the Ags can be quantitatively expressed as the association constant K_a (M^{-1}), or its reciprocal, the dissociation constant, K_d (M). K_d is equal to the concentration of free ligand at half-maximal complex formation. Alternatively, the thermodynamic equilibrium constants K_a and K_d can be obtained from the on- and off-rate constants: k_{on} and k_{off}, as indicated. Both the thermodynamic and the kinetic constants are relevant to explanations of neutralization. (b) The top row illustrates functional and intrinsic affinity and valency of Ab binding. The virion to the left has a bivalent IgG bound and the one to the right a Fab fragment of the same Ab. The IgG and Fab binding to soluble and immobilized Ag is shown to the right in the top row. The concentration of a monovalent soluble Ag that yields half-maximal binding to paratopes approximates the K_d relating to the *intrinsic* affinity, which would be expected to be the same for IgG and Fab binding. The concentration of a Fab that gives half-maximal binding on virion-associated and soluble Ag, respectively, may not be the same because of conformational differences between the two forms of the Ag: the intrinsic affinities of the paratopes for the two antigenic forms would then differ. More relevant to neutralization is the *functional* affinity of a whole bi-, tetra- or decavalent Ab for the epitopes in their context on the virions. The functional affinity simplifies matters by the postulate that the Ab is bound or unbound; although, for example, an IgG can have one or both paratopes bound. When both can bind, their doing so will be thermodynamically favoured and thereby the functional affinity will be substantially higher than the intrinsic affinity. This is the avidity effect, which might be quantified as the ratio of the functional over the intrinsic affinity (Klasse, 1996). Bivalent binding would also be possible when the monovalent Ag is immobilized onto a solid phase (shown top right). If the conformation and oligomeric state, and thereby the intrinsic affinity, as well as the spacing and orientation, of the Ag on the solid phase resemble those on the virion, then the functional affinity will be similar in the two situations. Below to the left, the potential effect of Ab hinge flexibility on functional affinity is shown. The flexibility of the hinge region of human IgG_3 is greater than that of the other IgG subclasses, which may facilitate two-point binding to a polyvalent Ag and increase the functional affinity of the Ab for the virion and thereby its neutralization potency (Cavacini *et al*., 1995; Scharf *et al*., 2001). In addition, as shown to the right, the Fc portions of neighbouring IgG molecules can interact non-covalently (Greenspan, 2001a), which could effectively double the valency of the Ab in the bound state. A neighbour effect of this kind will produce deviations from the law of mass action in Ab binding: intermolecular cooperativity may enhance the degree of binding achieved on the virion surface above certain Ab densities. (c) The qualitative aspect of affinity as structural complementarity between paratope and epitope is depicted schematically. Charge–charge interactions (+/−), hydrogen bonds (stippled lines) and van der Waals interactions make contributions to the binding energy. Affinity can be translated into changes in free energy, which relate to the system and not merely to the epitope and paratope (Greenspan & Cooper, 1995; Greenspan & Di Cera, 1999; Epa & Colman, 2001): the solvent (water molecules excluded from the molecular interface are schematically indicated), solutes, the energetics

that later event. According to this criterion, interference with release of progeny virus (Webster & Laver, 1967; Kilbourne *et al.*, 1968; Zebedee & Lamb, 1988; Mazanec *et al.*, 1995; Gerhard, 2001) should not be termed neutralization. The aggregation of virions by Abs can influence infectivity, in particular by reducing the number of plaques or foci in quantal infectivity assays. Although aggregation would thus fulfil an operational definition as stated above, it has been regarded as spurious neutralization (Mandel, 1978). Moreover, the possibility of partial neutralization by aggregation clashes with the view of neutralization as an all-or-nothing phenomenon (Burton *et al.*, 2001). We agree that an individual virion might be either infectious or not, although the average virion would have merely a reduced probability of infecting under certain conditions. Such absoluteness of the infectious state at a certain point in time would be possible even if the neutralization were reversible. However, the notion of absolute neutralization should perhaps be tempered in the light of experimental evidence. For example, virions that are seemingly neutralized at a low cell density can infect at higher cell densities (Layne *et al.*, 1991). Furthermore, the neutralization efficiency of particular Abs can differ between the types of target cell used (Grady & Kinch, 1985; Kjellén, 1985; Ruppach *et al.*, 2000). Increasing the sophistication with which neutralization is dissected will thus require corresponding improvements in the understanding of virus infectivity. In many infectivity assays the vast majority of virions that are potentially infectious will not infect (Kabat *et al.*, 1994); and although many virions that do not infect could be as infectious as those that do, the propensity to infect may vary over a broad spectrum. We suggest that neutralization therefore be conceived as a reduction in the infectious propensity to below the threshold of detection. This may avoid the impoverished statistical view while allowing for incremental (Icenogle *et al.*, 1983; Ruggeri & Greenberg, 1991; Schonning *et al.*, 1999) and catastrophic effects of Ab binding (Layne *et al.*, 1990; Ruggeri & Greenberg, 1991; Klasse & Moore, 1996). All-or-nothing neutralization would be empirically indistinguishable from the most extreme catastrophic model.

Antibody–virus binding

As a minimum requirement for neutralization, the paratopes (Ag-binding sites) of Abs must interact with epitopes on virion-associated Ags. The epitopes must be exposed on the surface of the virion, although exposure may be only transient or contingent upon interactions with receptors or other Abs (Kjellén & Pereira, 1968; Roivainen *et al.*, 1993; Thali *et al.*, 1993; Li *et al.*, 1994). In a structural perspective, epitopes and paratopes can be defined as an ensemble of atoms in the Ag and Ab, respectively, that are in contact with the other molecule in the complex. How well these surfaces fit is a qualitative aspect of affinity. For quantitative analyses of Ab–virus affinity, see Fig. 1.

During an Ab response, B-cells that express Ag receptors with the highest affinity for the Ag are clonally expanded. This kind of selection makes it implausible that important mechanisms of virus neutralization by Ab would require subtle conformational changes induced by the Ab (Burton *et al.*, 2001; Parren & Burton, 2001). Indeed, potently neutralizing Abs do not generally induce conformational changes in the Ag (Smith *et al.*, 1996; Che *et al.*, 1998; Hewat & Blaas, 2001). So-called 'hit-and-run' neutralization (Brioen *et al.*, 1985a) may be a rarity because of this: an Ab that readily dissociates after inducing conformational changes would have a lower affinity than one that stays bound. It should also be noted that if signals transmitted through specific modes of Ab binding were obligate events in neutralization, then neutralization-blocking Abs (Hashimoto & Prince, 1963; Massey & Schochetman, 1981a) would be a frequent occurrence, which does not seem to be the case (Klasse & Sattentau, 2001; Parren & Burton, 2001).

Over a wide range of virion concentrations, a given concentration of Ab neutralizes the same relative proportion of virus infectivity (Andrewes & Elford, 1933). This finding was referred to as the 'percentage law' but it is really the expected effect of Ab excess over Ag, which makes the fraction of complexed Ab negligible (Burnet *et al.*, 1937). Indeed, as described for human cytomegalovirus (HCMV), increasing the virion concentration 100-fold (keeping the infectious dose constant by adding inert particles) does not affect the titre of cross-neutralizing sera (Klein *et al.*, 1999). The explanation for this would be the relatively constant Ab occupancy on the virions. The relative occupancy of viral epitopes can be defined as the proportion ligated by a paratope. It is central to the analysis of neutralization that under ordinary assay conditions this relative occupancy is approximately determined by the total Ab concentration and its functional affinity for epitopes as presented on the virion (Fig. 1) (Klasse, 1996).

In a study of the Ab response during infection with VSV in

of solvation and of conformational states of bound and free Ag and Ab, as well as spatial context, all contribute (Greenspan, 2001b). Thus contact residues in epitope and paratope sometimes make smaller energetic contributions to the affinity than non-contact residues. Furthermore, residues in the paratope that are most crucial to the discrimination between different Ags may not make the greatest energetic contributions nor even be in contact with the epitope (Greenspan, 2001a, b). A residue in the epitope (*) crucial to Ab binding may be remote from the contact surfaces (Parry *et al.*, 1990). A subtle amino acid substitution that determines the sensitivity of HIV-1 to neutralization by certain Abs directed to the outer envelope glycoprotein, gp120 or SU, is located in gp41, the non-covalently associated transmembrane protein (TM) (Klasse *et al.*, 1993; Thali *et al.*, 1994). According to the three-dimensional structure of gp120 and knowledge of which parts of the molecule interact with gp41, the mutated residue would be distant from the epitopes (Kwong *et al.*, 1998).

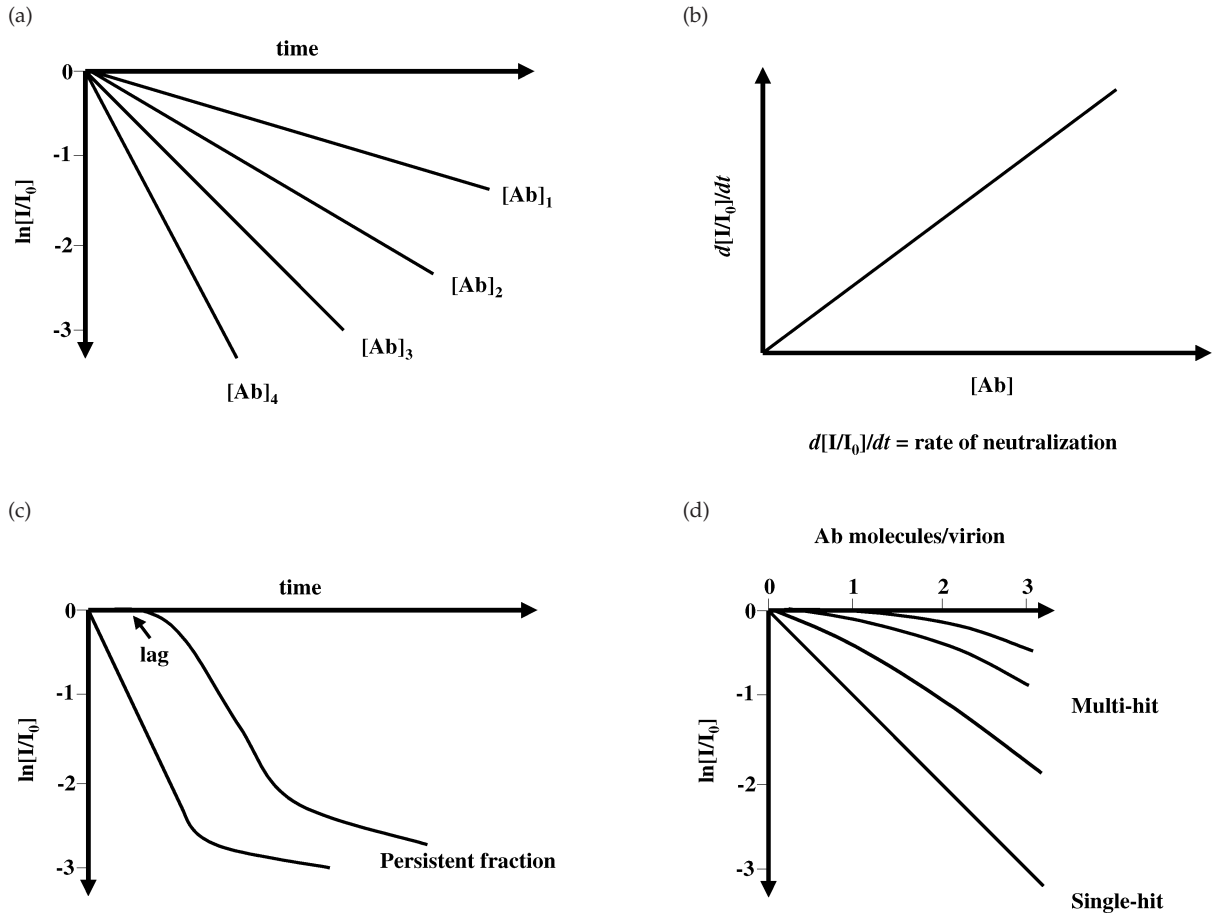

Fig. 2. The kinetics of neutralization. (a) The kinetic neutralization plot shows the logarithm of the fraction of residual infectivity (I/I_0) as a function of the time of Ab–virus incubation. The study of neutralization kinetics requires a method for quenching the reaction before addition of the Ab–virus mixture to susceptible cells. If the Ab–virus reaction is only negligibly reversible, or if Ab dissociation does not reverse neutralization, then dilution of the Ab–virus mixture can be such a method (Dulbecco *et al.*, 1956; Jerne & Avegno, 1956; Brioen *et al.*, 1985a; McDougal *et al.*, 1996). Linear curves for different Ab concentrations, increasing from $[Ab]_1$ to $[Ab]_4$, are shown. The rate of neutralization, $d\ln[I/I_0]/dt$, can thus be calculated for each Ab concentration. (b) When the rates of neutralization obtained from (a) are plotted as a function of Ab concentration and a linear relationship is obtained (as illustrated), this has been taken as evidence that the reaction is of first order in Ab concentration. But the molar excess of Ab over virus means that it is merely of pseudo-first order: determining the real order of the neutralization reaction would involve measurement of the concentration of uncomplexed Ab. Not even the true kinetics of the reaction would demonstrate the number of molecules involved, i.e. the *molecularity* of the reaction. (c) The common term 'single-hit kinetics' (not justified as a label for first-order, let alone pseudo-first-order, kinetics of the neutralization reaction) is sometimes applied to the lack of an initial shoulder or lag on the curve describing neutralization over time (Della-Porta & Westaway, 1978). The argument is that if the first Ab molecule knocks out the infectivity of the virion that it ligates, then there should be no lag, even at low temperatures and low Ab concentrations. Evidence for such lags has sometimes been overlooked in papers arguing for a single-hit mechanism (e.g. Dulbecco *et al.*, 1956). But it is uncertain whether absence of a lag could legitimately be used to support a single-hit hypothesis because of the comparatively rapid Ab binding, the difficulty in stopping and recording neutralization after a few seconds and, at least in the case of enveloped viruses, possible heterogeneity among virions, some of which may be neutralized more quickly than others (Klasse & Moore, 1996). A recurrent phenomenon is the levelling off of neutralization after a sharp drop in infectivity. The remaining non-neutralized virus is called *the persistent fraction*. Persistent infectivity, which can vary between different target cells (Kjellén, 1985), has been attributed to Ab dissociation and virus aggregation (reviewed in Burton *et al.*, 2001; Klasse & Sattentau, 2001). (d) The molecularity of virus neutralization by Ab corresponds to the minimum occupancy required for neutralization of the individual virion but does not follow directly from stoichiometrically determined average occupancy: when a subset of virions in a population mixed with Ab is neutralized, some of them will have greater than necessary occupancy. Occupancy can be analysed under the premise of Poisson-distributed Ab binding, as shown in the stoichiometric plot of the logarithm of relative infectivity as a function of the average number of Ab molecules per virion. If single-hit neutralization is assumed, then only virions with no Ab bound would be infectious. Single-hit neutralization corresponds to the linear curve that goes through the point (1, −1), i.e. 37% relative infectivity at the average of one Ab molecule per virion. That value, or preferably the whole curve, should be used to test the correspondence between the actual relative infectivity and the predicted one for single-hit neutralization. When a requirement for several Abs bound per virion has been shown (Flamand *et al.*, 1993; Icenogle *et al.*, 1983), the figure 37% is arbitrary. Each hypothetical multiplicity of hits should be tested by comparing its proper theoretical curve with the actual one (Klasse & Moore, 1996). Three non-linear, few-hit curves are plotted as examples.

mice, the neutralizing capacity of the sera was found to correlate better with the on-rate constant of binding (Fig. 1) than with affinity (Roost *et al*., 1995). Studies of human immunodeficiency virus type 1 (HIV-1) indicate that the on-rate of Ab binding to the oligomeric form of the envelope (Env) glycoprotein is more important with respect to neutralization than the affinity is (Fouts *et al*., 1997; Sattentau & Moore, 1995). The influence of the on-rate would also explain why neutralization is less potent the shorter the pre-incubation of Ab and virus is before mixing with the target cells (Burnet *et al*., 1937; Della-Porta & Westaway, 1978; McDougal *et al*., 1996). All of these findings are nevertheless consonant with the requirement of a certain Ab occupancy on the virions, whether at equilibrium or before, in order to abrogate infectivity. It was stated succinctly "That the 'survival' or 'inactivation' of a given virus particle is determined by the amount of antibody combined with it at the moment of effective contact with the susceptible cell" (Burnet *et al*., 1937). Because of the bound Ab, this effective contact may, of course, not arise; but it is important to take potential competition between Ab and receptors on the surface of the susceptible cell into account. And such competition would be subject to the influence of the kinetic constants of Ab binding to virus. However, much less emphasis has been placed on the potentially crucial rate constants of Ab and receptor binding by virus than on the kinetics of the neutralization reaction itself.

For nearly half a century, kinetic analyses of neutralization have dominated theories of how many Ab molecules must bind per virion in order to neutralize (Dulbecco *et al*., 1956). Recently, we have criticized the theoretical and empirical basis of these influential ideas (Klasse & Sattentau, 2001). The central arguments about kinetics, stoichiometry and occupancy are illustrated in Fig. 2.

Direct stoichiometric determinations of the number of Ab molecules on neutralized virions have generally corroborated multi-hit neutralization hypotheses. Poliovirus was shown to require an average of nine monoclonal Ab (mAb) molecules per virion for 90% of the infectivity to be neutralized (Icenogle *et al*., 1983) and a Poisson analysis of those data (Klasse & Moore, 1996) demonstrated a minimum of four or five molecules per virion for neutralization. In contrast, a study of polyclonal IgG neutralization of poliovirus suggested that virions with only one Ab molecule bound were non-infectious (Wetz *et al*., 1986). Rhinovirus, another picornavirus, requires 5–6 Ab molecules per virion for 50% and 10–20 Ab molecules for 90% neutralization (Smith *et al*., 1993). One report has suggested that a sucrose gradient-purified fraction of adenovirus with only 1·4 molecules of antihexon polyclonal IgG per virion was non-infectious (Wohlfart, 1988). This is surprising, since, according to a Poisson analysis, 25% of the virions would have no Ab bound. However, neutralization of adenovirus by Abs to the penton base requires full occupancy, i.e. on all five sites, which can be achieved by a Fab but not by the corresponding whole IgG (Stewart *et al*., 1997). This is not translatable into occupancy on the whole virion but would statistically require a large number of hits. A total of 36 mAb molecules binding monovalently to the capsomer tips of papillomavirus neutralized 50% of the infectivity. With a strikingly similar fraction of epitopes occupied, a mAb binding bivalently to the sides of the hexavalent capsomers neutralized virus by 50% when 14 Ab molecules were bound (Booy *et al*., 1998). Stoichiometric analyses indicate that 50% of influenza virus infectivity is neutralized when around 50 IgG molecules are bound, while 90% requires over 100 molecules (Taylor *et al*., 1987). This does not conflict with the kinetic data in the same study (Fig. 2). Neutralization of a majority of rabies virus infectivity requires from 130–350 IgG and 40–50 IgM molecules per virion (Flamand *et al*., 1993): the potential number of epitopes occupied would range from 260 to 700 for the bivalent IgG and from 400 to 500 for IgM. To conclude, where stoichiometric data are available, they generally indicate multi-hit neutralization. However, since molecularites, i.e. the required number of Ab molecules per virion, correspond to relative occupancies, single-hit neutralization can be refuted on approximate grounds in many other cases: when the Ab is in molar excess over the viral Ag and it is required at or above its K_d for binding to the virions, the relative occupancy could be estimated to be 50% or above (Klasse & Moore, 1996). This would correspond to several hits for symmetrical icosahedral or enveloped animal viruses, which, unlike some phage (Jerne & Avegno, 1956), generally have many copies of the neutralization Ag per virion.

Mechanisms of neutralization

Just as the kinetics of neutralization must not be confused with its molecularity or required occupancy, so it is imperative to keep the latter distinct from the mechanism by which Abs abrogate virus infectivity. Mechanism and occupancy may be linked but they must be analysed separately. Likewise, the question of when in the course of the infectivity assay the Ab must or can be present in order to neutralize is separate from the question of which replicative step it blocks. As illustrated in Fig. 3, mechanisms of neutralization can be classified according to the event in the virus replication cycle that they block (Mandel, 1978; Dimmock, 1984; Klasse *et al*., 1998; Klasse & Sattentau, 2001). We identify six such steps: virus attachment to target cells via receptors or ancillary molecules is a necessary first step in all virus replication. If it is blocked completely, then infection is effectively prevented. If the propensity of the virion to attach is merely reduced, then iterated encounters with susceptible cells will lead to infection. Aggregation can be a mechanism of neutralization in that it reduces the number of cells that virions can attach to. Potentially, however, the attachment of aggregates may be stronger and deliver a higher dose of virus to a smaller number of cells than monodisperse virions would. A second step with

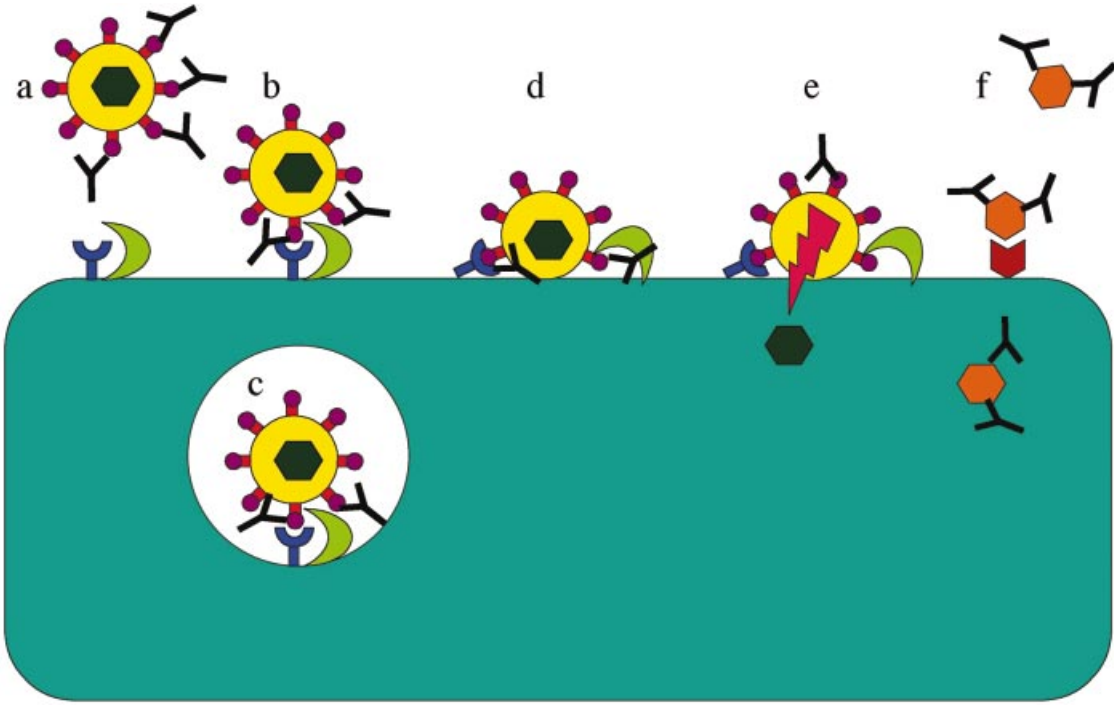

Fig. 3. Mechanisms of virus neutralization by Ab. (a) Ab binding to a proportion of the receptor-interactive structures on the virion may block virus attachment to the surface of target cells. In the schematic example shown, the Abs are bound to protein spikes on an enveloped virus, which is thereby prevented from making contact with either of the two cell-surface receptors that it uses for attachment and entry. (b) Ab inhibition of the interactions between the viral envelope protein and the cell-surface receptors is shown to occur after the virion has attached by binding via the receptors. For example, one receptor may serve as an attachment point as well as a trigger of conformational changes that allow interactions with a co-receptor, which in turn mediate later events such as membrane fusion. Ab interference with any of these necessary links in a chain of events that lead to entry would constitute a neutralization mechanism. (c) In order to infect, some viruses require internalization by endocytosis and the lowering of the pH in the endosome as a trigger of conformational changes in viral proteins. Abs that have not blocked virus attachment but allowed internalization are shown. Their block of requisite interactions between viral and cell-membrane proteins would delay or prevent the penetration of the viral core into the target-cell cytoplasm. The virion may thus ultimately be destroyed through lysosomal degradation. Such effects of Ab would constitute a mechanism of virus neutralization. In addition, Ab-mediated derouting of viruses that preferentially enter directly via the cell surface to a less permissive endosomal compartment may abrogate infectivity. (d) The intercalation of Ab in the fusion interface between the cell membrane and the envelope of a virus may block fusion at the cell surface, as illustrated, or in an endosome. (e) It has been conjectured that even a very low occupancy of antibody on the virion can cause global or internal changes by transmitting a signal across the viral envelope or outer layer. These hypothetical changes would allow the viral core to enter the cytoplasm but compromise further replicative steps. (f) The neutralization of naked viruses could potentially differ from that of enveloped viruses. Although naked viruses have been shown in several instances to be neutralized by a block of viral attachment to target cells, as in (a), conformational effects on the whole virion by Ab binding have also been registered as pI shifts. The possibility that the Ab–virion complex enters the cytoplasm and that the Ab blocks further replicative events is shown.

which Abs can interfere is provided by the post-attachment interactions of the virus with its receptors and co-receptors. A third possibility is that Abs on the virion may reduce its internalization by endocytosis. This would constitute neutralization when endocytosis is an obligate replicative step. If, however, endocytosis is a totally unproductive pathway, the derouting of virions into it, through Ab–Fc receptor interactions, could cause neutralization. A fourth stage of blocking is the fusion at the cell surface or in endosomal vesicles of enveloped viruses. Abs may interfere with the requisite fusogenic protein–protein interactions or with conformational changes that are instrumental to the fusion process. Alternatively, the Abs may simply obstruct contact between the two lipid membranes. For naked viruses, the corresponding membrane-penetration steps may be blocked. A fifth stage of interference is the uncoating or appropriate intracellular localization of core or capsid, which may hypothetically be affected by Abs. As a potential sixth step, if Abs that have bound to the virion surface could inhibit the first metabolic events catalysed by viral enzymes, such as transcription, this would count as neutralization.

In the former scheme, there are well-substantiated examples of neutralization by the first four mechanisms; there have been suggestions also of the last two, i.e. post-entry mechanisms, preferentially in conjunction with hypotheses of single- or few-hit molecularity (Fazekas de St Groth, 1962; Mandel, 1976; Dimmock, 1984). Bivalent Ab binding to the naked virions of poliovirus has been implicated in the blocking of

conformational changes, uncoating and conversion to a smaller form of the viral capsid (Mandel, 1976; Emini *et al.*, 1983; Wetz, 1993; Vrijsen, *et al.*, 1993). However, nAbs can also block attachment of naked viruses (Colonno *et al.*, 1989; Vrijsen *et al.*, 1993; Verdaguer *et al.*, 1997; Booy *et al.*, 1998) and the capacity to induce conformational changes in the virion correlates poorly with neutralizing efficiency (Che *et al.*, 1998; Smith, 2001). Some Abs will block poliovirus attachment if allowed to bind to the virus before the addition to cells; if added afterwards, the same Abs will block a later replicative step (Vrijsen *et al.*, 1993). Post-entry effects, even interference with transcription, by nAbs to enveloped viruses have been suggested (Possee *et al.*, 1982; Armstrong & Dimmock, 1992; Armstrong *et al.*, 1996; McInerney *et al.*, 1997) but would require as yet unexplained transmembrane signalling events (Burton *et al.*, 2001; Klasse & Sattentau, 2001). We emphasize that, according to the definition, interference with any of these steps would qualify as neutralization. Experiment alone can determine which mechanisms really do occur. Furthermore, although unity is conceptually attractive, there is no theoretical reason to rule out the existence of multifarious neutralization mechanisms among distinct Abs in their prevention of different viruses from infecting a variety of target cells.

Interference with virus attachment and virus–receptor interactions

Virus attachment is necessary for infection: blocking that step pre-empts the entry process. This mechanism is important in the neutralization of many naked and enveloped viruses (reviewed by Burton *et al.*, 2001; Klasse & Sattentau, 2001; Parren & Burton, 2001; Smith, 2001). Certain Abs prevent the extracellular enveloped form of vaccinia virus from attaching to target cells (Law & Smith, 2001). Likewise, several Abs to gp120, the outer Env protein of HIV-1 (Fig. 4a), block attachment of the virus to $CD4^+$ T-cell lines regardless of whether they are directed to epitopes directly involved in interactions with CD4, the primary receptor for the virus (Ugolini *et al.*, 1997). However, a mAb to the transmembrane protein, gp41, recognizing intact Env spikes, neutralizes well without any detectable effect on virus attachment. This differential effect by the generally attachment-blocking gp120 mAbs and the gp41 mAb that allows neutralized virus to attach to cells could be attributed to differences in the proximity of the bound mAb to the viral membrane and in the angle of binding to different components of the Env protein spikes (Figs 3 and 4). A comparison could be made with two mAbs to papillomavirus, which show differential effects on virus attachment to cells: one binds bivalently and deeply between hexavalent capsomeres and only partially blocks attachment, whereas the other mAb binds monovalently to the outer surface of pentavalent capsomeres and completely blocks virus attachment (Booy *et al.*, 1998). Abs to different sites on La Crosse virus prevent attachment to cells in various degrees, which only partly explains their neutralizing capacity (Kingsford *et al.*, 1991). Likewise, nAbs to different Ags on the surface of rotavirus inhibit virus attachment to varying extents (Ruggeri & Greenberg, 1991).

The potent and broad capacity of sera from HIV-1-infected people to block HIV-1 infection of peripheral blood mononuclear cells has also been attributed to inhibition of virus attachment to the cell surface (Beirnaert *et al.*, 2001). However, another study observed nAb-mediated inhibition of attachment only to immortalized and not to primary T-cells (Spenlehauer *et al.*, 2001). Furthermore, different nAbs to HIV-1 gp120 have distinct and sometimes opposing effects on HIV attachment depending on the type of target cell (Mondor *et al.*, 1998b). Thus, a block of attachment may explain neutralization only in some of these cases.

Neutralization epitopes on the influenza virus haemagglutinin (HA) surround the receptor-binding pocket. This suggests that neutralization interferes with receptor binding and that mutations at these sites may abrogate Ab binding but not the docking of the receptor into its pocket (Skehel & Wiley, 2000). For some viruses, the situation is more complicated: in these instances, different viral receptors are required for attachment to the cell surface and entry. Certain viruses, including herpes simplex virus (WuDunn & Spear, 1989) and foot-and-mouth disease virus (FMDV) (Fry *et al.*, 1999), make use of ancillary receptors, such as heparan sulphate proteoglycans, for initial tethering to the target cell; they rely on other molecules for internalization or triggering of fusion (Ugolini *et al.*, 1999). Some viruses, e.g. adenovirus and HIV, interact with co-receptors as a step in the entry process. All of these interactions are potential targets for nAbs, which may interfere with the attachment process in various degrees. Indeed, most HIV-1 gp120-specific nAbs block either CD4–gp120 binding or co-receptor–gp120 binding, or both (Mondor *et al.*, 1998a; Trkola *et al.*, 1996; Wu *et al.*, 1996). The Env glycoprotein of bovine leukaemia virus, a deltaretrovirus, is prevented from binding to a recombinant form of the receptor for the virus, BLVRcp1, by sera from infected cattle but not by some mAbs to Env (Orlik *et al.*, 1997). It is uncertain how either kind of Ab would affect virus attachment to permissive cells.

We have given several examples of nAbs that act by blocking virus attachment to cells after Ab–virus pre-incubation and some nAbs that do not. Such effects are independent of a capacity of certain Abs for blocking infection of virus that has already absorbed to target cells (Mandel, 1978; Armstrong *et al.*, 1992; Lu *et al.*, 1992; Vrijsen *et al.*, 1993; Pelchen-Matthews *et al.*, 1995; Armstrong *et al.*, 1996; McInerney *et al.*, 1997; Edwards & Dimmock, 2001a). This capacity does not imply that the Abs block the same step at physiological temperature or when present before the virus–cell encounter: several Abs that can block attachment are also capable of neutralizing after the virus has adsorbed to cells at a temperature that does not allow entry. Furthermore, it should

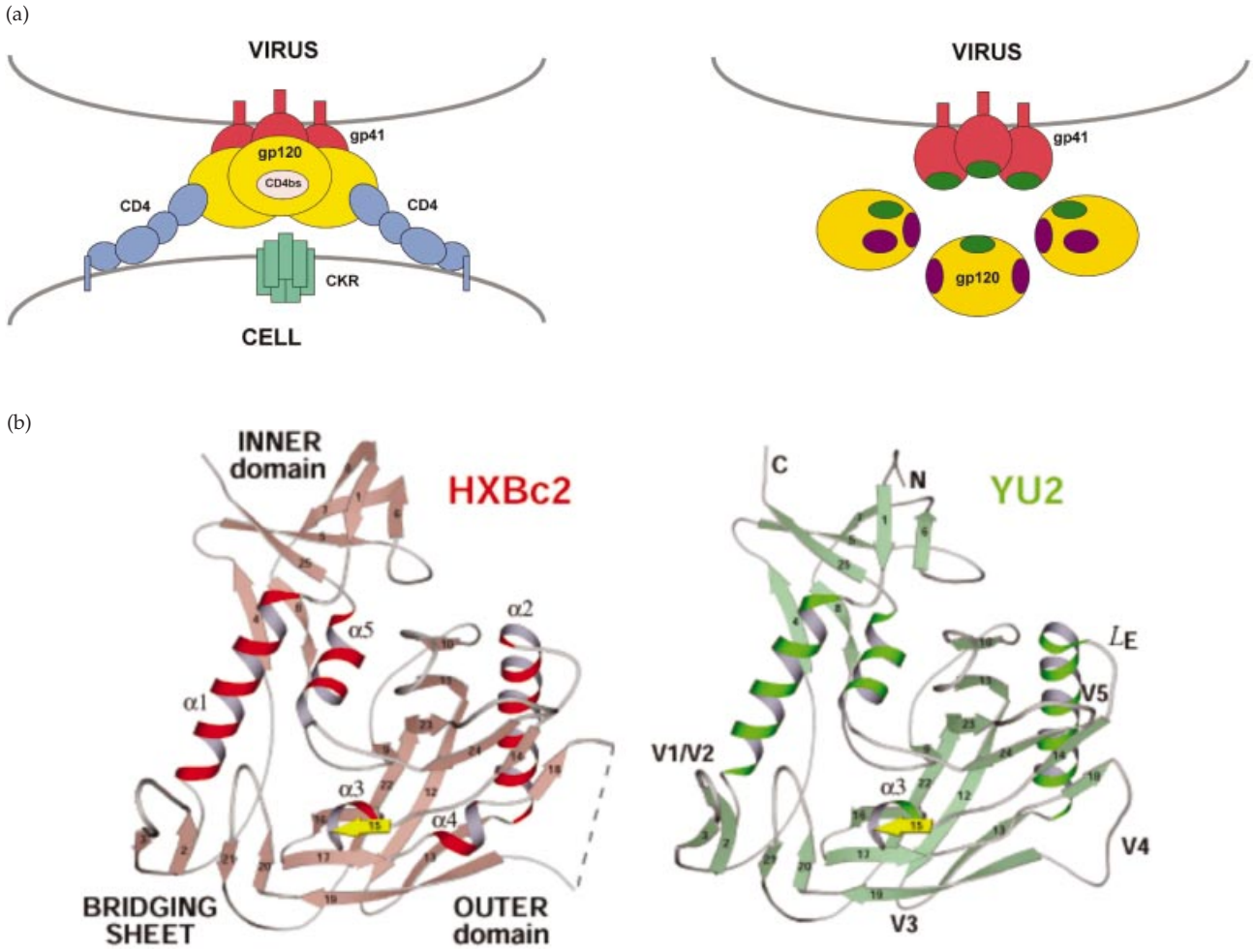

Fig. 4. For legend see facing page.

be addressed experimentally whether Abs added after virus attachment can bring about the detachment of pre-adsorbed virions from target cells (Dietzschold *et al.*, 1987; Ruggeri & Greenberg, 1991; Edwards & Dimmock, 2001a): i.e. does post-attachment neutralization act by reversing attachment?

In conclusion, although many Abs interfere both with virus–receptor interactions and with attachment, particular Abs that block receptor interactions may neutralize without blocking virus attachment to certain cell types, whereas others that do not directly interfere with receptor binding of viral proteins block attachment of virions to cells (Massey & Schochetman, 1981b; Linsley *et al.*, 1988; McInerney *et al.*, 1997; Ugolini *et al.*, 1997; Mondor *et al.*, 1998b; Klasse & Sattentau, 2001; Smith, 2001). Interference with receptor interactions subsequent to virus attachment can also effect neutralization (Armstrong & Dimmock, 1996; Armstrong *et al.*, 1996; Lu *et al.*, 1992). Regardless of this, when blockage of attachment does occur, whether through direct occlusion of sites involved in virus binding to cells or indirectly through steric interference by Ab coating of the virions, then infection is by necessity prevented.

Induction of conformational changes by nAbs

The binding of some Abs to the capsid of picornaviruses results in conformational changes, which are reflected in a shift in the isoelectric pH, the pI, of the virions (Che *et al.*, 1998; Mandel, 1976, 1978; Smith, 2001). However, this effect does not correlate with the extent of neutralization (Brioen *et al.*, 1985b); the pI shift is not a prerequisite for the neutralization of rhinoviruses (Smith, 2001). Rather, whereas the paratope of one strongly neutralizing Fab undergoes significant conformational changes when it binds, the epitope itself is left essentially unchanged (Smith *et al.*, 1996). Furthermore, although Abs to four different neutralization epitopes on the rhinovirus capsid effect the pI changes when they bind, they are unlikely to cause similar conformational changes because of their disparate sites and modes of binding (Colonno *et al.*, 1989). They would be most likely to affect the conformation of distinct, flexible loop regions and not the protein core. Abs to picornaviruses can stabilize virions against changes induced by low pH and such changes may be necessary for infection via the endosome. But these stabilizing

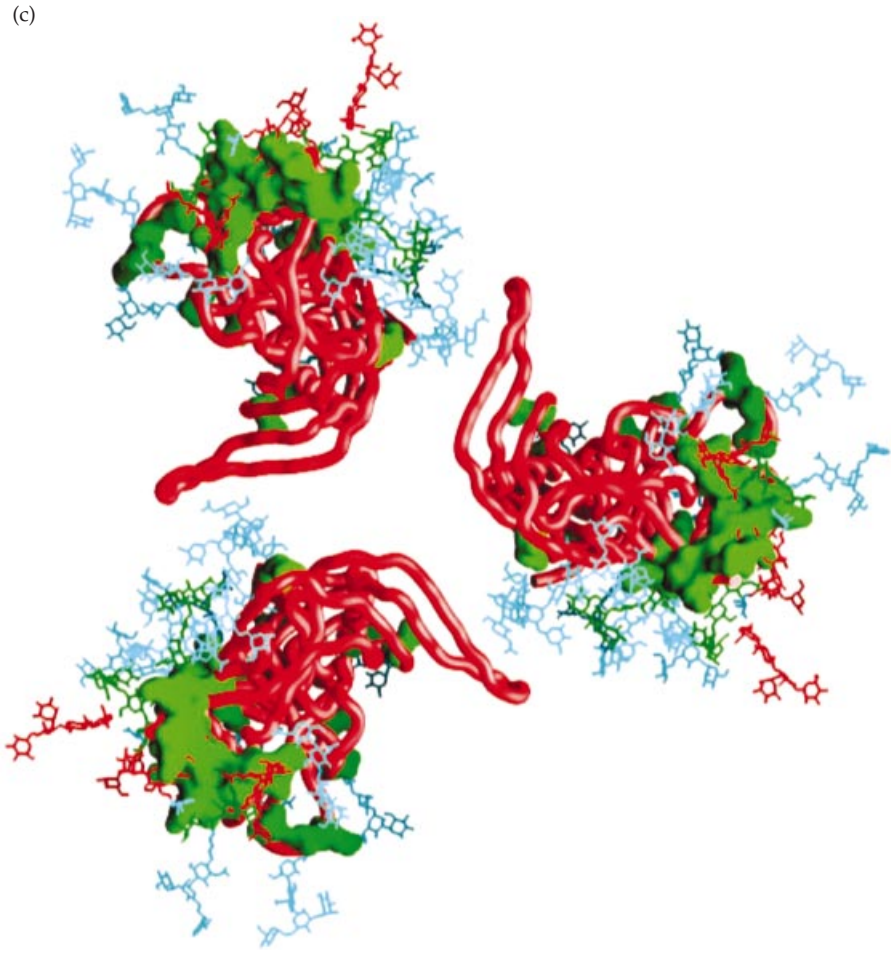

Fig. 4. The structure of a virus neutralization Ag. (a) Schematic model of the quaternary structure of the HIV-1 Env glycoprotein. The surrounding envelope of a virion of a TCLA strain is studded with approximately 70 such Env spikes (Grewe *et al*., 1990). A spike is thought to consist of a trimer (Chan *et al*., 1997; Weissenhorn *et al*., 1997) of heterodimers, each of which comprises the outer surface unit (SU), gp120, anchored non-covalently to the transmembrane protein (TM), gp41. The interactions between such an oligomeric spike and the primary receptor, the four-domain CD4 molecule, on the target cell membrane is indicated. CD4-induced conformational changes are conducive to interactions with a chemokine receptor that eventually promote fusion of the two membranes. Neutralization may break distinct links in this chain of events (left). The quaternary-structural arrangement indicates that a number of epitopes on both SU and TM are occluded completely or unavailable for antibody binding through steric hindrance due to the TM–TM and SU–TM (green) interactions or SU–SU contiguity (purple). Abs binding to epitopes that are only exposed on the soluble monomeric form of SU and those that only recognize the uncomplexed TM generally do not neutralize (Burton & Parren, 2000; Burton *et al*., 2000; Moore *et al*., 2001) (right). (b) Primary isolates of HIV-1 are considerably more resistant to neutralization than TCLA strains. Ribbon diagrams show the crystallographically determined tertiary core structures of an SU of a TCLA strain (HXBc2) and of a PI strain (YU2), in which the hypervariable regions V1/V2 and V3 are truncated. The interaction with TM covers an area around the N and C termini at the top of the inner domain. The major receptor, CD4, binds in the crevice between the inner and outer domains, adjacent to the bridging sheet. The co-receptor-binding site is induced by CD4 and involves the bridging sheet and the V3 region. The receptor-interactive surfaces overlap with important neutralization epitopes. The mixed β-sheet/α-helix and supersecondary structure of the two domains with their connection through the bridging sheet is superimposable for the two SUs (for details see Kwong *et al*., 2000a). This similarity of the TCLA and PI core SU structures suggests that the cause of the substantially greater neutralization sensitivity of TCLA than PI strains is to be found instead in the influence on antigenicity of the variable regions and of the quaternary structure, or in the minimal occupancy required for neutralization. (Kwong *et al*., 2000a). (c) Comparison of HIV-1 PI and TCLA SU molecules as oriented in a modelled envelope-glycoprotein trimer (Kwong *et al*., 2000b). The C α worm of the TCLA SU, including modelled variable regions, is shown in red. The core of the PI SU is superimposed, with sequence differences marked in green. The cores of the carbohydrate chains common to both SUs are shown in cyan; those specific to the TCLA strain in red and to the PI strain in green. The oligomer is seen from the target cell membrane with the TM anchorage below the plane of the picture. The differences in the sequences of the cores of the subunits and in the glycosylation patterns may influence the antigenicity of neutralization epitopes; however, the degree of tightness or openness of the trimer is likely to have an overriding influence on Ab binding and hence on neutralization sensitivity. Courtesy of Peter Kwong and with permission from Elsevier Science (Kwong *et al*., *Structure* 8, 1329–1339, 2000).

Abs, whether aggregating or not, neutralize rhinovirus to widely differing extents (Che *et al.*, 1998). Moreover, Abs of other specificities do not block pH-induced changes, yet neutralize well. It therefore seems telling that Abs to all the rhinovirus neutralization epitopes are instead capable of preventing cellular attachment of the virus (Colonno *et al.*, 1989), thus blocking a step prior to internalization and pH-induced changes. The hypothesis of neutralization through capsid stabilization implies that some mutations would mediate escape from neutralization by specifically preventing such stabilizing effects. Yet all the neutralization-escape mutants of rhinovirus that have been analysed show impaired Ab binding compared with wild-type (Smith, 2001). Hence, conformational changes induced by nAbs may constitute dispensable epiphenomena.

The study of HIV-1 provides some examples of Ab-induced conformational changes that are not necessary for neutralization but may nevertheless affect it. One of the most potent nAbs (IgG_1 b12), which is directed against the outer Env protein gp120, binds to an epitope that overlaps with the CD4-binding site (Burton *et al.*, 1994). Whereas CD4 induces conformational changes in gp120 (Moore *et al.*, 1990), the Ab does not (Poignard *et al.*, 1996) but rather appears to recognize an epitope that is present on the native conformation of the antigen (Saphire *et al.*, 2001). However, several other Abs to gp120 affect the conformation of the Env complex when they bind, as demonstrated by their induction of gp120 shedding from the transmembrane glycoprotein, gp41 (Fig. 4a) (Poignard *et al.*, 1996). When such shedding occurs, the number of functional Env oligomers on the virion decreases. There is evidence that spontaneous dissociation of gp120 makes the virus more sensitive to neutralization by soluble CD4 (Layne *et al.*, 1990), as explained by an occupancy model of neutralization (Klasse & Moore, 1996). However, the model predicts that the same degree of neutralization would be achieved by a higher-affinity Ab that does not induce dissociation (Fig. 5b). A complication is that the indirect steric effect of a bound Ab would disappear with the Ag, thus giving a weaker neutralizing effect on neighbouring spikes than an Ab that does not induce shedding. The net effect cannot be calculated on the basis of our present knowledge.

High-occupancy theories of neutralization

Single- and few-hit theories of neutralization imply that specific effects of Ab binding are somehow transmitted to unoccupied viral surface Ag molecules or that the requisite functions of the interior of the virions are compromized by Abs that bind to their exterior. We have reviewed how these theories can be criticized on the basis of physico-chemical, immunological and virological analyses of neutralization. An alternative conjecture is that Abs act primarily by virtue of their *binding*, because each virion needs a certain number of unimpeded functional surface Ag molecules in order to infect. When Ab binding leaves fewer such Ag copies unobstructed than the virus needs, then it is neutralized (Klasse & Moore, 1996). It should be noted that the requirement for a certain minimal occupancy of Ab on the virion would allow that lower occupancies enhance infectivity through conformational triggering of entry functions or through complement- or Fc receptor-dependent mechanisms (for a recent review, see Sullivan, 2001).

A meticulous investigation of rabies virus neutralization provides an example of multiple-hit molecularity. Neutralization of rabies virus requires occupancy by more than 200 IgG or 40 IgM molecules per virion (Flamand *et al.*, 1993). An average of 130 IgG or 30 IgM per virion gives little or no neutralization; 37% relative infectivity (an arbitrary figure for these multi-hit molecularities) is obtained at 130–320 IgG or 40–50 IgM. In relative terms, these levels of Ab binding would correspond to occupancies of $> 30\%$ for IgG and 50% for IgM, defined as proportions of actual binding over maximal possible binding (i.e. saturation). According to another definition of relative occupancy, i.e. the fraction of epitopes occupied by paratopes, the maximum relative occupancy can then be well below 100% because steric hindrance may prevent some epitopes from being occupied at saturation. The gap between the two definitions begs the question whether maximal binding of Ab can leave such a high fraction of unoccupied epitopes (because of steric constraints) that it is non-neutralizing. The adenovirus fibre Ag may cause steric hindrance of this kind: the significant proportion of unoccupied penton-base epitopes at saturating Ab concentrations could explain the lack of neutralization by certain Abs (Stewart *et al.*, 1997; Stewart & Nemerow, 1997). This would also account for the unusual situation that the Fab neutralizes while the IgG does not (Fig. 1b). A more complex and intriguing relationship between IgG and Fab neutralization has been described for influenza virus: in one case, an IgG neutralizes partly by blocking virus attachment and more efficiently than its Fab, which only prevents fusion, although they have similar functional affinities for the HA Ag. In other cases, the Fabs are found to neutralize exclusively by blocking attachment, while the corresponding IgGs interfere both with attachment and with fusion. Nevertheless, all are capable of preventing infection after virus adsorption (Edwards & Dimmock, 2000, 2001a, b). Future studies may specifically dissect the effects of valency and Ab bulk on binding and interference with attachment and fusion or penetration of different viruses.

The study of rabies virus provides another important test case for neutralization hypotheses: Abs that specifically recognize a minority conformation of spike proteins do not neutralize at physiological pH. However, if all of the spike protein is converted to this antigenic conformation by a lowering of the pH, then the Abs neutralize (Flamand *et al.*, 1993; Raux *et al.*, 1995). This refutes the simplistic view that all Abs that can bind to functional virus surface molecules involved in entry or attachment will necessarily neutralize: it

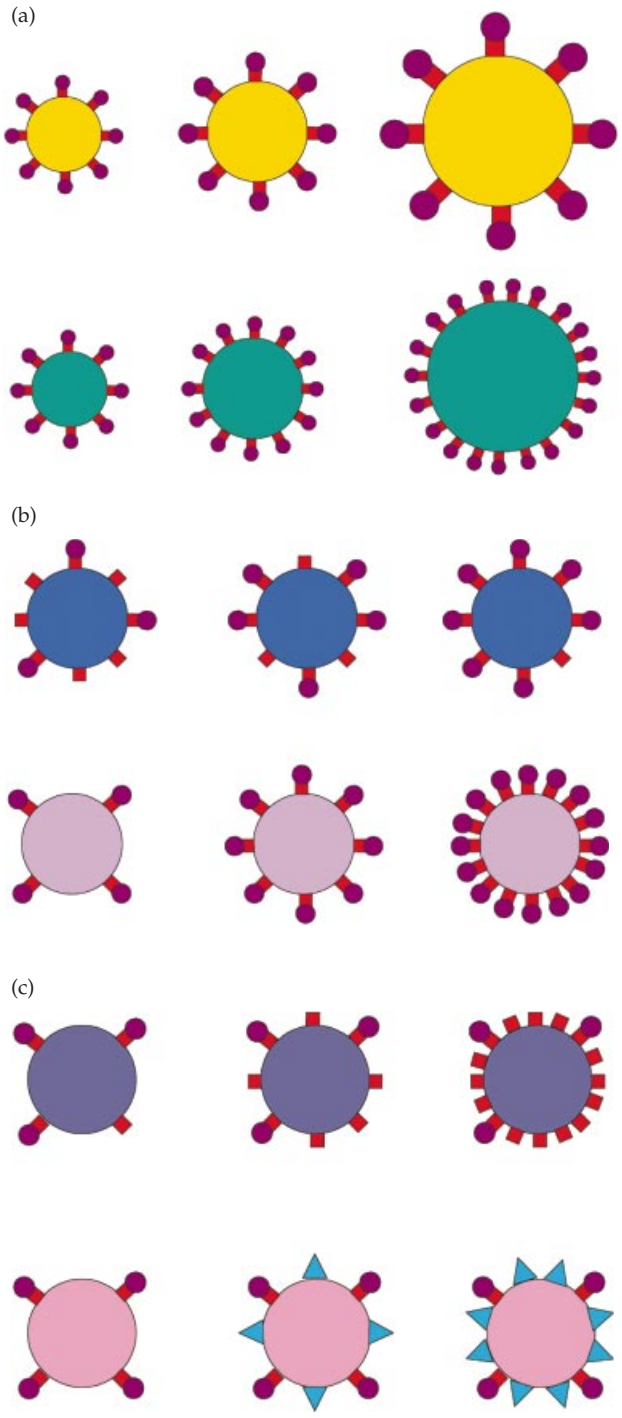

Fig. 5. Precise test implications of occupancy theories of neutralization must take into account the size of the virion, the antigenic surfaces of the viral receptor-binding structures, the number of such sites, their functionality, their proximity to each other and to viral or non-viral Ags that do not mediate virus entry. The Ab occupancy on virions is determined by the ratio of Ab concentration over the dissociation constant (K_d) for binding to the respective epitope as presented on the virion: the effects of such occupancy would be the net result of all the factors above. For purposes of illustration, the model virions have spikes that stick out from the virion surface, somewhat reminiscent of enveloped viruses. But the principles would apply equally well to naked viruses. (a) The three different viruses in the upper row have the same number of spikes per virion but with increasing virion size, the spike size increases too. The differences could have several consequences for sensitivity to neutralization. One Ab bound to one spike on the smallest virion may have some steric-blocking effect on a neighbouring spike, while such an effect would be weaker on the larger virions. Furthermore, a large spike may require several Abs bound in order to be completely prevented from functioning in attachment and entry. For the virions in the lower row, the spike number increases with increasing virion size. If the same minimum number of functional spikes are needed for the different virions in order to infect, a greater proportion would then be occupied on the larger than the smaller virions. (b) The upper row represents a virus that loses an outer spike protein, like some strains of HIV, thereby rendering the spikes inert. If the three virions require the same number of intact, functional spikes for infection, the minimal neutralizing occupancy would increase from left to right. The virions in the lower row do not lose spikes through shedding but have had increasing numbers of spikes incorporated at budding or assembly from left to right. They could represent different virus species or mutants of the same virus. Unlike in (a) the virions are of the same size. Just as for the row above, if the minimal number of functional spikes for infection were constant, a greater obstruction on the virions to the right would be required. Potential enhancement of two-point binding, however, would increase the functional affinity and thereby give higher occupancy at a given Ab concentration than for the virions towards the left. More efficient indirect steric hindrance might tend to decrease the minimal occupancy required for neutralization. (c) In the upper row three virions of the same size with a constant number of functional spikes are represented. The outer spike protein is lost to various degrees and the number of inert spikes created by this shedding rises from left to right. The coating theory of neutralization would predict that as the density of non-functional spikes increases, Abs that bind only to them would become neutralizing. The explanation for why Abs that bind to TM after SU shedding from HIV particles do not neutralize would therefore be that the TM density is too low for these Abs to interfere with the function of the intact spikes. The lower row represents virions of constant size with the same number of functional spikes. In addition, another protein is present at increasing densities on the virions to the right. This could be, e.g. viral NA on influenza virions or a cellular Ag on HIV particles. The coating theory implies that if such a non-entry or non-viral Ag is present at a certain density, then Abs binding to it will neutralize by sterically blocking receptor access to the functional spikes (see text).

suggests instead that a certain minimum proportion of such molecules must be blocked for neutralization to occur.

Neutralization by antibody coating

Burton and colleagues (Burton *et al.*, 2001; Parren & Burton, 2001) have investigated the relationship between the surface area on virions and the number of Ab molecules required for neutralization, where these data can be extracted with some confidence from the literature. For five different viruses, and even when the larger, non-virus pathogen *Chlamydia trachomatis* is included, there is an approximately linear relationship between the number of nAbs required to neutralize the majority of the infectivity and the particle surface area. This supports the coating theory proposed by the authors: neutralization of viruses is due to steric or direct blocking of a

proportion of the virion surface such that the requisite interactions between virus and the cellular membrane are prevented.

The coating theory explains why Abs to non-viral molecules on the virion surface can neutralize infectivity. It has interesting implications also for the incorporation into viral envelopes of viral proteins that are not required for infection. For example, Abs to influenza virus neuraminidase (NA) and the M2 protein do not neutralize but block release of progeny virions (Webster & Laver, 1967; Kilbourne *et al.*, 1968; Zebedee & Lamb, 1988). Does the demonstration of Abs that bind to these Ags on the virion surface but still do not neutralize refute the coating theory of neutralization? As pointed out recently, the receptor-binding and fusogenic HA protein is vastly more abundant than either NA or M2 on the virion surface; furthermore, NA is unevenly distributed over the virion surface (Parren & Burton, 2001). Therefore, the maximal occupancy on these Ags may not be able to cause steric interference with entry functions. However, the coating theory implies that at a higher density of NA molecules, such Abs would become neutralizing (Fig. 5c); likewise, a potentiating effect of NA Abs on neutralization by HA Abs could be predicted. One can ask whether the density of viral Ags that do not mediate entry is kept down by a selective pressure.

An illustrative example of the effects of non-viral, non-essential molecules is the incorporation of the intercellular adhesion molecule 1 (ICAM-1) in HIV-1 particles (Rizzuto & Sodroski, 1997). The presence of ICAM-1 enhances infection of $CD4^+$ T-cells; it makes the virus less sensitive to anti-Env neutralization and highly susceptible to an Ab against ICAM-1. The reduction in infectivity by the ICAM-1 Ab is several-fold greater than the enhancement of infectivity by the presence of ICAM-1. Thus, the ICAM-1 Ab interferes not just with an auxiliary ICAM-1 function in infectivity but collaterally with Env function. Furthermore, truncation of the cytoplasmic tail of simian immunodeficiency virus Env reduces incorporation of HLA-I, -II and co-expressed influenza virus HA; neutralization by Abs to these non-HIV molecules decreases accordingly (Vzorov & Compans, 2000). This substantiates the coating theory, which implies that a dense coat of Ab over the virion surface will interfere by steric hindrance with the function of unoccupied viral molecules.

Neutralization of bovine papillomavirus by different Abs requires different occupancies (Roden *et al.*, 1994). This is attributable to differences in the distribution of sites over the virion surface, mono- or bivalent binding and the angle of projection from the virion of the bound Ig molecule. Furthermore, one-point binding in a protruding manner coincides with blocking of virus attachment, while bivalent binding that is more flush with the virion surface neutralizes at least partly by a post-attachment mechanism (Booy *et al.*, 1998). Such subtle effects of valency and orientation of bound Ab (Hewat & Blaas, 1996; Hewet *et al.*, 1998) may explain why there are deviations from linearity in the plot of the minimum number of Abs per virion required for neutralization (Burton *et al.*, 2001). But if the area of virion surface that is occluded by Ab could be measured for different modes of Ab binding, then the area–stoichimetric relationship would be testable with some precision. A refinement of the high-occupancy or coating theory could be formulated thus: neutralization occurs when the number of unencumbered entry-mediating viral molecules is brought below a required minimum by Ab occupancy. This would imply only approximate linearity in the plot of the number of Ab molecules needed as a function of virion area. As structural and stoichiometric data for more viruses become available, it will be possible to test whether the number of functional entry-mediating viral protein units per virion correlates better than does virion area with the minimal number of neutralizing Ab molecules (Fig. 5).

It has been suggested previously that occupancy of one monomeric subunit by one Ab paratope may be sufficient to knock out the function of the entire HIV Env trimer and potentially also that of adjacent trimers (Klasse & Moore, 1996). If there is a minimum number of necessary Ag molecules required for infection, then the higher their density, the greater the minimal occupancy needed for neutralization (Fig. 5). In the case of HIV, this seems to be the case (Layne *et al.*, 1990): shedding of the outer Env protein gp120 increases neutralization sensitivity. The tendency of some strains of HIV-1 to shed their Env (Moore *et al.*, 1990) could provide a testing ground for refinement of the coating theory. When the outer Env protein, gp120, is shed, it converts a functional spike to an inert one while exposing epitopes on the transmembrane protein, gp41. Although these epitopes are strongly immunogenic in natural infection, Abs directed to them are generally not neutralizing or only weakly so (Gnann *et al.*, 1987; Ho *et al.*, 1987; Bugge *et al.*, 1990; McDougal *et al.*, 1996; Verrier *et al.*, 2001; Wilson *et al.*, 1990). The coating theory explains this on the basis that the density of transmembrane protein spikes on the virion is too low to achieve a neutralizing Ab coat. Alternatively, the lack of neutralization could be due to the functional inertia of such spikes in combination with their too long distance from functional spikes. Thus, with increased shedding (as long as it remains compatible with infectivity), neutralization by these gp41 Abs could become significant if non-specific coating were responsible; or, on the contrary, there would be no added effect, if proximity to functional spikes were insufficient. Abs to gp120 would decrease in potency with shedding because of less general coating and neighbour blocking effects; or, alternatively, their potency would increase because the required occupancy would be lower when the virions have fewer 'spare' spikes (Fig. 5). This example illustrates that the coating theory can be formulated in more precise versions with distinct test implications.

There are qualitative complications of steric hindrance by coating: the angle and valency of Ab binding, as well as the bulkiness of the Fc portion, can influence the area of virion surface that is blocked. The mobility of the epitope and of the

Ab bound to it might influence the size of the virion-surface area that is prevented from functional interactions. However, two mAbs that bind to the same highly mobile loop on FMDV, one in a fixed manner, the other allowing loop flexibility, neutralize the virus with indistinguishable efficiency (Hewat *et al.*, 1997; Verdaguer *et al.*, 1999). A mAb that binds human rhinovirus bivalently with a maximum of 30 molecules per virion neutralizes the virus to a great extent; other nAbs that bind monovalently at a maximum of 60 molecules per virion neutralize and aggregate the virus to highly divergent degrees (Smith *et al.*, 1993; Smith, 2001). These differences are not readily attributable to variable functional affinities since they occur at saturating mAb concentrations. Neutralization of papillomavirus to half-maximal extent by one bivalently and one monovalently binding mAb requires the occupancy of similar numbers of epitopes and roughly twice as many molecules of the latter as of the former (Booy *et al.*, 1998). Once the functional affinity factor is controlled for by comparison of similar actual occupancies, a prediction from the coating theory could be that the thicker coat of monovalently bound antibody would neutralize to a greater extent. Since this is not the case, perhaps dynamic factors such as stability of Ab binding and competition by cellular receptors may have to be invoked in a satisfactory occupancy theory of neutralization.

Although aggregation is not a universal effect of the binding of efficient nAbs (Che *et al.*, 1998), it can qualify as a mechanism of neutralization. Therefore it is pertinent here to point out its relationship to occupancy. Aggregation requires at least bivalent antibody binding that links virus particles into lattices. Such cross-linking typically occurs at intermediate Ab–virus ratios, so that the prevalence of non-aggregated virus describes a U-shaped relationship to Ab concentration. A corresponding U-shaped virus survival curve would refute the coating theory, since non-aggregation at higher Ab concentrations is explained by saturation of all epitopes by monovalently binding Ab. As the aggregates are broken up by excess Ab, however, infectivity sometimes increases but is not completely restored (Thomas *et al.*, 1986; Smith, 2001). The coating theory thus survives this test.

Antibody coating and virus escape

Can the modes of virus escape refute or corroborate the coating theory of neutralization? A high-occupancy theory implies that a decrease in the affinity of the nAb for the target antigen will readily mediate virus evasion. This is indeed a prevalent mode of escape (reviewed by Smith, 2001; Bizebard *et al.*, 2001). If the affinity is not reduced, neutralization insensitivity could instead be due to an increased difference between the total and minimally required numbers of functional viral surface proteins involved in attachment and entry functions (Klasse & Moore, 1996). Predicted escape mechanisms derived from the non-specific coating effect would be a decrease in the density of Ab binding sites or a change in the mode of Ab binding, such that a smaller area of the viral surface is blocked sterically at a certain occupancy. A neutralization-escape mutant of HCMV may be a case in point. The escape phenotype coincides with a reduction in the number of copies of the gH protein on the viral surface. In the absence of selection, there is swift reversion to neutralization sensitivity (Li *et al.*, 1995). It would be crucial to determine whether the mutant has switched from dependence on gH for mediating entry.

In the field of HIV research, what might be understood as the reverse of escape from neutralization has come to the fore. Primary isolates (PI) of HIV are substantially more resistant to neutralization than T-cell line-adapted (TCLA) strains obtained by reiterated passage *in vitro* (Moore & Ho, 1995). One factor that determines this difference may be the absence of nAbs *in vitro*: their presence *in vivo* may constitute a selective pressure that maintains the resistant phenotype. Other characteristics of the *in vitro* growth conditions may select for phenotypic traits that entail the neutralization-resistant phenotype as a mere contingency. In either case, resistance may be mediated by a relative inaccessibility of the neutralization epitopes on the Env oligomers as presented on the virion surface (Fig. 4) (Parren & Burton, 2001; Poignard *et al.*, 1996; Sattentau *et al.*, 1999). Such relative inaccessibility would simply reduce the functional affinities of the Abs for their epitopes. Thereby only subneutralizing occupancies might be achieved at ordinary Ab concentrations. The explanation is consonant with a requirement for high occupancy and hence with the coating theory.

Some mAbs that do not neutralize rabies virus under ordinary conditions will do so at lower pH, at higher temperature or after prolonged incubation. These conditions presumably allow the sufficiently prevalent adoption of antigenic conformations of the relevant epitopes. Mutants resistant to this special neutralization have been selected (Raux *et al.*, 1995). The mechanism of resistance may be prevention of a conformational change in the majority of the molecules or it may make the changed conformation non-antigenic. In either case, an occupancy of too few spikes on the mutant virus for neutralization to occur would explain the resistance (Raux *et al.*, 1995).

However, another neutralization escape mutant of rabies virus poses a further challenge to the coating theory. Although this mutant can bind over 1000 IgG molecules per virion, it is still completely resistant to neutralization (Flamand *et al.*, 1993). Whether the Ag to which the Ab binds still performs its function in attachment and entry or the mutant has adopted alternative strategies is unknown. It also remains to be determined whether the few unoccupied epitopes at Ab saturation are somehow more accessible and functional than on wild-type virus, whether the orientation of bound Ab is affected by the escape mutation and whether a kinetic difference in binding allows receptor competition with Ab on the mutant. Answers to these questions could either refute or corroborate differentiated hypotheses of neutralization as

mediated by general coating of virions or, alternatively, by specific interference with the function of Ags involved in virus entry and attachment. However, it should be remembered that this potential exception to the general rule that high occupancy effects neutralization is a viral mutant and not a naturally occurring virus.

Conclusion

The task of eliciting an efficient nAb response would be simplified if the goal were maximal concentration and affinity for virions of the Abs rather than their capacity to induce specific, subtle conformational changes and thereby to neutralize by intricate mechanisms. Nevertheless, escape from neutralization by different Abs with similar affinities but qualitatively distinct modes of binding may occur with varying molecular propensities.

Theoretical and empirical support is strong for inhibition of virus attachment and early entry functions as dominant mechanisms of neutralization. Likewise, multi-hit molecularities of neutralization, as well as viral escape through mutations that decrease Ab affinity, are well substantiated for several viruses. Occupancy of Ab on the viral surface is thus central to theories of neutralization. High occupancy of Abs on viral or other Ags may form blocking coats around the virion. Future research may elucidate the requirements for the density of the Abs and for their contiguity to functionally crucial sites on the virion. An improved understanding of neutralization and virus escape from it will inform the selection and modification of immunogens in vaccine design with the aim of eliciting optimal nAb responses.

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References

Andrewes, C. H. & Elford, W. J. (1933). Observations on anti-phage sera. I. 'The percentage law'. *British Journal of Experimental Pathology* **14**, 367–376.

Armstrong, S. J. & Dimmock, N. J. (1992). Neutralization of influenza virus by low concentrations of hemagglutinin-specific polymeric immunoglobulin A inhibits viral fusion activity, but activation of the ribonucleoprotein is also inhibited. *Journal of Virology* **66**, 3823–3832.

Armstrong, S. J. & Dimmock, N. J. (1996). Varying temperature-dependence of post-attachment neutralization of human immunodeficiency virus type 1 by monoclonal antibodies to gp120: identification of a very early fusion-independent event as a neutralization target. *Journal of General Virology* **77**, 1397–1402.

Armstrong, S. J., McInerney, T. L., McLain, L., Wahren, B., Hinkula, J., Levi, M. & Dimmock, N. J. (1996). Two neutralizing anti-V3 monoclonal antibodies act by affecting different functions of human immunodeficiency virus type 1. *Journal of General Virology* **77**, 2931–2941.

Bachmann, M. F., Kundig, T. M., Kalberer, C. P., Hengartner, H. & Zinkernagel, R. M. (1994). How many specific B cells are needed to protect against a virus? *Journal of Immunology* **152**, 4235–4241.

Bachmann, M. F., Kalinke, U., Althage, A., Freer, G., Burkhart, C., Roost, H., Aguet, M., Hengartner, H. & Zinkernagel, R. M. (1997). The role of antibody concentration and avidity in antiviral protection. *Science* **276**, 2024–2027.

Beirnaert, E., De Zutter, S., Janssens, W. & van der Groen, G. (2001). Potent broad cross-neutralizing sera inhibit attachment of primary HIV-1 isolates (groups m and o) to peripheral blood mononuclear cells. *Virology* **281**, 305–314.

Bizebard, T., Berbey-Martin, C., Fleury, D., Gigant, B., Barrere, B., Skehel, J. J. & Knossow, M. (2001). Structural studies on viral escape from antibody neutralization. *Current Topics in Microbiology and Immunology* **260**, 55–64.

Booy, F. P., Roden, R. B., Greenstone, H. L., Schiller, J. T. & Trus, B. L. (1998). Two antibodies that neutralize papillomavirus by different mechanisms show distinct binding patterns at 13 Å resolution. *Journal of Molecular Biology* **281**, 95–106.

Brioen, P., Rombaut, B. & Boeye, A. (1985a). Hit-and-run neutralization of poliovirus. *Journal of General Virology* **66**, 2495–2499.

Brioen, P., Thomas, A. A. & Boeye, A. (1985b). Lack of quantitative correlation between the neutralization of poliovirus and the antibody-mediated pI shift of the virions. *Journal of General Virology* **66**, 609–613.

Bugge, T. H., Lindhardt, B. O., Hansen, L. L., Kusk, P., Hulgaard, E., Holmback, K., Klasse, P. J., Zeuthen, J. & Ulrich, K. (1990). Analysis of a highly immunodominant epitope in the human immunodeficiency virus type 1 transmembrane glycoprotein, gp41, defined by a human monoclonal antibody. *Journal of Virology* **64**, 4123–4129.

Burnet, F. M., Keogh, E. V. & Lush, D. (1937). The immunological reactions of the filterable viruses. *Australian Journal of Experimental Biology and Medical Science* **15**, 227–368.

Burton, D. R. & Parren, P. W. (2000). Vaccines and the induction of functional antibodies: time to look beyond the molecules of natural infection? *Nature Medicine* **6**, 123–125.

Burton, D. R., Pyati, J., Koduri, R., Sharp, S. J., Thornton, G. B., Parren, P. W., Sawyer, L. S., Hendry, R. M., Dunlop, N., Nara, P. L. and others (1994). Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science* **266**, 1024–1027.

Burton, D. R., Williamson, R. A. & Parren, P. W. (2000). Antibody and virus: binding and neutralization. *Virology* **270**, 1–3.

Burton, D. R., Saphire, E. O. & Parren, P. W. (2001). A model for neutralization of viruses based on antibody coating of the virion surface. *Current Topics in Microbiology and Immunology* **260**, 109–143.

Cavacini, L. A., Emes, C. L., Power, J., Desharnais, F. D., Duval, M., Montefiori, D. & Posner, M. R. (1995). Influence of heavy chain constant regions on antigen binding and HIV-1 neutralization by a human monoclonal antibody. *Journal of Immunology* **155**, 3638–3644.

Chan, D. C., Fass, D., Berger, J. M. & Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–273.

Che, Z., Olson, N. H., Leippe, D., Lee, W. M., Mosser, A. G., Rueckert, R. R., Baker, T. S. & Smith, T. J. (1998). Antibody-mediated neutralization of human rhinovirus 14 explored by means of cryoelectron microscopy and X-ray crystallography of virus–Fab complexes. *Journal of Virology* **72**, 4610–4622.

Colonno, R. J., Callahan, P. L., Leippe, D. M., Rueckert, R. R. & Tomassini, J. E. (1989). Inhibition of rhinovirus attachment by neutralizing monoclonal antibodies and their Fab fragments. *Journal of Virology* **63**, 36–42.

Della-Porta, A. J. & Westaway, E. G. (1978). A multi-hit model for the neutralization of animal viruses. *Journal of General Virology* **38**, 1–19.

Dietzschold, B., Tollis, M., Lafon, M., Wunner, W. H. & Koprowski, H. (1987). Mechanisms of rabies virus neutralization by glycoprotein-specific monoclonal antibodies. *Virology* **161**, 29–36.

Dimmock, N. J. (1984). Mechanisms of neutralization of animal viruses. *Journal of General Virology* **65**, 1015–1022.

Dulbecco, R., Vogt, M. & Strickland, A. G. R. (1956). A study of the basic aspects of neutralization. Two animal viruses: Western equine encephalitis virus and poliomyelitis virus. *Virology* **2**, 162–205.

Edwards, M. J. & Dimmock, N. J. (2000). Two influenza A virus-specific Fabs neutralize by inhibiting virus attachment to target cells, while neutralization by their IgGs is complex and occurs simultaneously through fusion inhibition and attachment inhibition. *Virology* **278**, 423–435.

Edwards, M. J. & Dimmock, N. J. (2001a). Hemagglutinin 1-specific immunoglobulin G and Fab molecules mediate postattachment neutralization of influenza A virus by inhibition of an early fusion event. *Journal of Virology* **75**, 10208–10218.

Edwards, M. J. & Dimmock, N. J. (2001b). A haemagglutinin (HA1)-specific Fab neutralizes influenza A virus by inhibiting fusion activity. *Journal of General Virology* **82**, 1387–1395.

Emini, E. A., Ostapchuk, P. & Wimmer, E. (1983). Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. *Journal of Virology* **48**, 547–550.

Epa, V. C. & Colman, P. M. (2001). Shape and electrostatic complementarity at viral antigen–antibody complexes. *Current Topics in Microbiology and Immunology* **260**, 45–53.

Fazekas de St Groth, S. (1962). The neutralization of viruses. *Advances in Virus Research* **9**, 1–125.

Flamand, A., Raux, H., Gaudin, Y. & Ruigrok, R. W. (1993). Mechanisms of rabies virus neutralization. *Virology* **194**, 302–313.

Fouts, T. R., Binley, J. M., Trkola, A., Robinson, J. E. & Moore, J. P. (1997). Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. *Journal of Virology* **71**, 2779–2785.

Fry, E. E., Lea, S. M., Jackson, T., Newman, J. W., Ellard, F. M., Blakemore, W. E., Abu-Ghazalaleh, R., Samuel, A., King, A. M. & Stuart, D. I. (1999). The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. *EMBO Journal* **18**, 543–554.

Gerhard, W. (2001). The role of the antibody response in influenza virus infection. *Current Topics in Microbiology and Immunology* **260**, 171–190.

Gnann, J. W., Jr, Nelson, J. A. & Oldstone, M. B. (1987). Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. *Journal of Virology* **61**, 2639–2641.

Grady, L. J. & Kinch, W. (1985). Two monoclonal antibodies against La Crosse virus show host-dependent neutralizing activity. *Journal of General Virology* **66**, 2773–2776.

Greenspan, N. S. (2001a). Affinity, complementarity, cooperativity, and specificity in antibody recognition. *Current Topics in Microbiology and Immunology* **260**, 65–85.

Greenspan, N. S. (2001b). Dimensions of antigen recognition and levels of immunological specificity. *Advances in Cancer Research* **80**, 147–187.

Greenspan, N. S. & Cooper, L. J. (1995). Complementarity, specificity and the nature of epitopes and paratopes in multivalent interactions. *Immunology Today* **16**, 226–230.

Greenspan, N. S. & Di Cera, E. (1999). Defining epitopes: it's not as easy as it seems. *Nature Biotechnology* **17**, 936–937.

Grewe, C., Beck, A. & Gelderblom, H. R. (1990). HIV: early virus–cell interactions. *Journal of Acquired Immune Deficiency Syndromes* **3**, 965–974.

Hashimoto, N. & Prince, A. M. (1963). Kinetic studies on the neutralization reaction between Japanese encephalitis virus and antiserum. *Virology* **19**, 261–272.

Hewat, E. A. & Blaas, D. (1996). Structure of neutralizing antibody bound bivalently to human rhinovirus 2. *EMBO Journal* **15**, 1515–1523.

Hewat, E. & Blaas, D. (2001). Structural studies on antibody interacting with viruses. *Current Topics in Microbiology and Immunology* **260**, 29–44.

Hewat, E. A., Verdaguer, N., Fita, I., Blakemore, W., Brookes, S., King, A., Newman, J., Domingo, E., Mateu, M. G. & Stuart, D. I. (1997). Structure of the complex of a Fab fragment of a neutralizing antibody with foot-and-mouth disease virus: positioning of a highly mobile antigenic loop. *EMBO Journal* **16**, 1492–1500.

Hewat, E. A., Marlovits, T. C. & Blaas, D. (1998). Structure of a neutralizing antibody bound monovalently to human rhinovirus 2. *Journal of Virology* **72**, 4396–4402.

Ho, D. D., Sarngadharan, M. G., Hirsch, M. S., Schooley, R. T., Rota, T. R., Kennedy, R. C., Chanh, T. C. & Sato, V. L. (1987). Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. *Journal of Virology* **61**, 2024–2028.

Icenogle, J., Shiwen, H., Duke, G., Gilbert, S., Rueckert, R. & Anderegg, J. (1983). Neutralization of poliovirus by a monoclonal antibody: kinetics and stoichiometry. *Virology* **127**, 412–425.

Jerne, N. K. & Avegno, P. (1956). The development of the phage-inactivating properties of serum during the course of specific immunization of an animal: reversible and irreversible inactivation. *Journal of Immunology* **76**, 200–208.

Kabat, D., Kozak, S. L., Wehrly, K. & Chesebro, B. (1994). Differences in CD4 dependence for infectivity of laboratory-adapted and primary patient isolates of human immunodeficiency virus type 1. *Journal of Virology* **68**, 2570–2577.

Kilbourne, E. D., Laver, W. G., Schulman, J. L. & Webster, R. G. (1968). Antiviral activity of antiserum specific for an influenza virus neuraminidase. *Journal of Virology* **2**, 281–288.

Kingsford, L., Boucquey, K. H. & Cardoso, T. P. (1991). Effects of specific monoclonal antibodies on La Crosse virus neutralization: aggregation, inactivation by Fab fragments, and inhibition of attachment to baby hamster kidney cells. *Virology* **180**, 591–601.

Kjellén, L. (1985). A hypothesis accounting for the effect of the host cell on neutralization-resistant virus. *Journal of General Virology* **66**, 2279–2283.

Kjellén, L. & Pereira, H. G. (1968). Role of adenovirus antigens in the induction of neutralizing antibody. *Journal of General Virology* **2**, 177–185.

Klasse, P. J. (1996). Physico-chemical analysis of the humoral immune response to HIV-1: quantification of antibodies, their binding to viral antigens and neutralization of viral infectivity. In *HIV Molecular Immunology Database*, part IV, pp. 22–52. Edited by B. Korber, B. Walker, R. Koup, J. Moore, B. Haynes & G. Myers. Los Alamos, New Mexico: Los Alamos National Laboratory, Theoretical Biology and Biophysics.

Klasse, P. J. & Moore, J. P. (1996). Quantitative model of antibody- and soluble CD4-mediated neutralization of primary isolates and T-cell line-adapted strains of human immunodeficiency virus type 1. *Journal of Virology* **70**, 3668–3677.

Klasse, P. J. & Sattentau, Q. J. (2001). Mechanisms of virus neutralization by antibodies. *Current Topics in Microbiology and Immunology* **260**, 87–108.

Klasse, P. J., McKeating, J. A., Schutten, M., Reitz, M. S., Jr & Robert-Guroff, M. (1993). An immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 [HXB2-Env: Ala(→ Thr)] decreases viral neutralization by monoclonal antibodies to the CD4-binding site. *Virology* **196**, 332–337.

Klasse, P. J., Bron, R. & Marsh, M. (1998). Mechanisms of enveloped virus entry into animal cells. *Advanced Drug Delivery Reviews* **34**, 65–91.

Klein, M., Schoppel, K., Amvrossiadis, N. & Mach, M. (1999). Strain-specific neutralization of human cytomegalovirus isolates by human sera. *Journal of Virology* **73**, 878–886.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659.

Kwong, P. D., Wyatt, R., Majeed, S., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (2000a). Structures of HIV-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates. *Structure, Folding and Design* **8**, 1329–1339.

Kwong, P. D., Wyatt, R., Sattentau, Q. J., Sodroski, J. & Hendrickson, W. A. (2000b). Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. *Journal of Virology* **74**, 1961–1972.

Law, M. & Smith, G. L. (2001). Antibody neutralization of the extracellular enveloped form of vaccinia virus. *Virology* **280**, 132–142.

Layne, S. P., Merges, M. J., Dembo, M., Spouge, J. L. & Nara, P. L. (1990). HIV requires multiple gp120 molecules for CD4-mediated infection. *Nature* **346**, 277–279.

Layne, S. P., Merges, M. J., Spouge, J. L., Dembo, M. & Nara, P. L. (1991). Blocking of human immunodeficiency virus infection depends on cell density and viral stock age. *Journal of Virology* **65**, 3293–3300.

Li, Q., Yafal, A. G., Lee, Y. M., Hogle, J. & Chow, M. (1994). Poliovirus neutralization by antibodies to internal epitopes of VP4 and VP1 results form reversible exposure of these sequences at physiological temperature. *Journal of Virology* **68**, 3965–3970.

Li, L., Coelingh, K. L. & Britt, W. J. (1995). Human cytomegalovirus neutralizing antibody-resistant phenotype is associated with reduced expression of glycoprotein H. *Journal of Virology* **69**, 6047–6053.

Linsley, P. S., Ledbetter, J. A., Kinney-Thomas, E. & Hu, S. L. (1988). Effects of anti-gp120 monoclonal antibodies on CD4 receptor binding by the Env protein of human immunodeficiency virus type 1. *Journal of Virology* **62**, 3695–3702.

Lu, S., Putney, S. D. & Robinson, H. L. (1992). Human immunodeficiency virus type 1 entry into T cells: more-rapid escape from an anti-V3 loop than from an antireceptor antibody. *Journal of Virology* **66**, 2547–2550.

McDougal, J. S., Kennedy, M. S., Orloff, S. L., Nicholson, J. K. A. & Spira, T. J. (1996). Mechanisms of human immunodeficiency virus type 1 (HIV-1) neutralization: irreversible inactivation of infectivity by anti-HIV-1 antibody. *Journal of Virology* **69**, 5236–5245.

McInerney, T. L., McLain, L., Armstrong, S. J. & Dimmock, N. J. (1997). A human IgG_1 (b12) specific for the CD4-binding site of HIV-1 neutralizes by inhibiting the virus fusion entry process, but b12 Fab neutralizes by inhibiting a postfusion event. *Virology* **233**, 313–326.

Mandel, B. (1976). Neutralization of poliovirus: a hypothesis to explain the mechanism and the one-hit character of the neutralization reaction. *Virology* **69**, 500–510.

Mandel, B. (1978). Neutralization of animal viruses. *Advances in Virus Research* **23**, 205–268.

Massey, R. J. & Schochetman, G. (1981a). Viral epitopes and monoclonal antibodies: isolation of blocking antibodies that inhibit virus neutralization. *Science* **213**, 447–449.

Massey, R. J. & Schochetman, G. (1981b). Topographical analysis of viral epitopes using monoclonal antibodies: mechanism of virus neutralization. *Virology* **115**, 20–32.

Mazanec, M. B., Coudret, C. L. & Fletcher, D. R. (1995). Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. *Journal of Virology* **69**, 1339–1343.

Mondor, I., Moulard, M., Ugolini, S., Klasse, P. J., Hoxie, J., Amara, A., Delaunay, T., Wyatt, R., Sodroski, J. & Sattentau, Q. J. (1998a). Interactions among HIV gp120, CD4, and CXCR4: dependence on CD4 expression level, gp120 viral origin, conservation of the gp120 COOH- and NH_2-termini and V1/V2 and V3 loops, and sensitivity to neutralizing antibodies. *Virology* **248**, 394–405.

Mondor, I., Ugolini, S. & Sattentau, Q. J. (1998b). Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. *Journal of Virology* **72**, 3623–3634.

Moore, J. P. & Ho, D. D. (1995). HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells. *AIDS* **9**, S117–S136.

Moore, J. P., McKeating, J. A., Weiss, R. A. & Sattentau, Q. J. (1990). Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* **250**, 1139–1142.

Moore, J. P., Parren, P. W. & Burton, D. R. (2001). Genetic subtypes, humoral immunity, and human immunodeficiency virus type 1 vaccine development. *Journal of Virology* **75**, 5721–5729.

Nowak, M. A. & Bangham, C. R. (1996). Population dynamics of immune responses to persistent viruses. *Science* **272**, 74–79.

Orlik, O., Ban, J., Hlavaty, J., Altaner, C., Kettmann, R., Portetelle, D. & Splitter, G. A. (1997). Polyclonal bovine sera but not virus-neutralizing monoclonal antibodies block bovine leukemia virus (BLV) gp51 binding to recombinant BLV receptor BLVRcp1. *Journal of Virology* **71**, 3263–3267.

Parren, P. W. & Burton, D. R. (2001). The antiviral activity of antibodies *in vitro* and *in vivo*. *Advances in Immunology* **77**, 195–262.

Parry, N., Fox, G., Rowlands, D., Brown, F., Fry, E., Acharya, R., Logan, D. & Stuart, D. (1990). Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. *Nature* **347**, 569–572.

Pelchen-Matthews, A., Clapham, P. & Marsh, M. (1995). Role of CD4 endocytosis in human immunodeficiency virus infection. *Journal of Virology* **69**, 8164–8168.

Phillips, A. N. (1996). Reduction of HIV concentration during acute infection: independence from a specific immune response. *Science* **271**, 497–499.

Poignard, P., Fouts, T., Naniche, D., Moore, J. P. & Sattentau, Q. J. (1996). Neutralizing antibodies to human immunodeficiency virus type-1 gp120 induce envelope glycoprotein subunit dissociation. *Journal of Experimental Medicine* **183**, 473–484.

Possee, R. D., Schild, G. C. & Dimmock, N. J. (1982). 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. *Journal of General Virology* **58**, 373–386.

Raux, H., Coulon, P., Lafay, F. & Flamand, A. (1995). Monoclonal antibodies which recognize the acidic configuration of the rabies glycoprotein at the surface of the virion can be neutralizing. *Virology* **210**, 400–408.

Rizzuto, C. D. & Sodroski, J. G. (1997). Contribution of virion ICAM-1 to human immunodeficiency virus infectivity and sensitivity to neutralization. *Journal of Virology* **71**, 4847–4851.

Roden, R. B., Weissinger, E. M., Henderson, D. W., Booy, F., Kirnbauer, R., Mushinski, J. F., Lowy, D. R. & Schiller, J. T. (1994). Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. *Journal of Virology* **68**, 7570–7574.

Roivainen, M., Piirainen, L., Rysa, T., Narvanen, A. & Hovi, T. (1993). An immunodominant N-terminal region of VP1 protein of poliovirus that is buried in the crystal structure can be exposed in solution. *Virology* **195**, 762–765.

Roost, H. P., Bachmann, M. F., Haag, A., Kalinke, U., Pliska, V., Hengartner, H. & Zinkernagel, R. M. (1995). Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proceedings of the National Academy of Sciences, USA* **92**, 1257–1261.

Ruggeri, F. M. & Greenberg, H. B. (1991). Antibodies to the trypsin cleavage peptide VP8 neutralize rotavirus by inhibiting binding of virions to target cells in culture. *Journal of Virology* **65**, 2211–2219.

Ruppach, H., Nara, P., Raudonat, I., Elanjikal, Z., Rubsamen-Waigmann, H. & Dietrich, U. (2000). Human immunodeficiency virus (HIV)-positive sera obtained shortly after seroconversion neutralize autologous HIV type 1 isolates on primary macrophages but not on lymphocytes. *Journal of Virology* **74**, 5403–5411.

Saphire, E. O., Parren, P. W., Pantophlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R. & Wilson, I. A. (2001). Crystal structure of a neutralizing human IgG against HIV-1: a template for vaccine design. *Science* **293**, 1155–1159.

Sattentau, Q. J. & Moore, J. P. (1995). Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. *Journal of Experimental Medicine* **182**, 185–196.

Sattentau, Q. J., Moulard, M., Brivet, B., Botto, F., Guillemot, J. C., Mondor, I., Poignard, P. & Ugolini, S. (1999). Antibody neutralization of HIV-1 and the potential for vaccine design. *Immunology Letters* **66**, 143–149.

Scharf, O., Golding, H., King, L. R., Eller, N., Frazier, D., Golding, B. & Scott, D. E. (2001). Immunoglobulin G3 from polyclonal human immunodeficiency virus (HIV) immune globulin is more potent than other subclasses in neutralizing HIV type 1. *Journal of Virology* **75**, 6558–6565.

Schønning, K., Lund, O., Lund, O. S. & Hansen, J. E. (1999). Stoichiometry of monoclonal antibody neutralization of T-cell line-adapted human immunodeficiency virus type 1. *Journal of Virology* **73**, 8364–8370.

Skehel, J. J. & Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual Review of Biochemistry* **69**, 531–569.

Smith, T. J. (2001). Antibody interactions with rhinovirus: lessons for mechanisms of neutralization and the role of immunity in viral evolution. *Current Topics in Microbiology and Immunology* **260**, 1–29.

Smith, T. J., Olson, N. H., Cheng, R. H., Hansong, L., Chase, E. S., Lee, W. M., Leippe, D. M., Mosser, A. G., Rueckert, R. R. & Baker, T. S. (1993). Structure of human rhinovirus complexed with Fab fragments from a neutralizing antibody. *Journal of Virology* **67**, 1148–1158.

Smith, T. J., Chase, E. S., Schmidt, T. J., Olson, N. H. & Baker, T. S. (1996). Neutralizing antibody to human rhinovirus 14 penetrates the receptor-binding canyon. *Nature* **383**, 350–354.

Spenlehauer, C., Kirn, A., Aubertin, A. M. & Moog, C. (2001). Antibody-mediated neutralization of primary human immunodeficiency virus type 1 isolates: investigation of the mechanism of inhibition. *Journal of Virology* **75**, 2235–2245.

Stewart, P. L. & Nemerow, G. R. (1997). Recent structural solutions for antibody neutralization of viruses. *Trends in Microbiology* **5**, 229–233.

Stewart, P. L., Chiu, C. Y., Huang, S., Muir, T., Zhao, Y., Chait, B., Mathias, P. & Nemerow, G. R. (1997). Cryo-EM visualization of an exposed RGD epitope on adenovirus that escapes antibody neutralization. *EMBO Journal* **16**, 1189–1198.

Sullivan, N. J. (2001). Antibody-mediated enhancement of viral disease. *Current Topics in Microbiology and Immunology* **260**, 145–169.

Taylor, H. P., Armstrong, S. J. & Dimmock, N. J. (1987). Quantitative relationships between an influenza virus and neutralizing antibody. *Virology* **159**, 288–298.

Thali, M., Moore, J. P., Furman, C., Charles, M., Ho, D. D., Robinson, J. & Sodroski, J. (1993). Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *Journal of Virology* **67**, 3978–3988.

Thali, M., MacArthur, C., Furman, C., Cavacini, L., Posner, M., Robinson, J. & Sodroski, J. (1994). Resistance to neutralization by broadly reactive antibodies to the human immunodeficiency virus type 1 gp120 glycoprotein conferred by a gp41 amino acid change. *Journal of Virology* **68**, 674–680.

Thomas, A. A., Vrijsen, R. & Boeye, A. (1986). Relationship between poliovirus neutralization and aggregation. *Journal of Virology* **59**, 479–485.

Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J. & Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384**, 184–187.

Ugolini, S., Mondor, I., Parren, P. W., Burton, D. R., Tilley, S. A., Klasse, P. J. & Sattentau, Q. J. (1997). Inhibition of virus attachment to $CD4^+$ target cells is a major mechanism of T cell line-adapted HIV-1 neutralization. *Journal of Experimental Medicine* **186**, 1287–1298.

Ugolini, S., Mondor, I. & Sattentau, Q. J. (1999). HIV-1 attachment: another look. *Trends in Microbiology* **7**, 144–149.

Verdaguer, N., Fita, I., Domingo, E. & Mateu, M. G. (1997). Efficient neutralization of foot and mouth virus disease virus by monovalent antibody binding. *Journal of Virology* **71**, 9813–9816.

Verdaguer, N., Schoehn, G., Ochoa, W. F., Fita, I., Brookes, S., King, A., Domingo, E., Mateu, M. G., Stuart, D. & Hewat, E. A. (1999). Flexibility of the major antigenic loop of foot-and-mouth disease virus bound to a Fab fragment of neutralising antibody: structure and neutralisation. *Virology* **255**, 260–268.

Verrier, F., Nadas, A., Gorny, M. K. & Zolla-Pazner, S. (2001). Additive effects characterize the interaction of antibodies involved in neutralization of the primary dualtropic human immunodeficiency virus type 1 isolate 89.6. *Journal of Virology* **75**, 9177–9186.

Vrijsen, R., Mosser, A. & Boeye, A. (1993). Postabsorption neutralization of poliovirus. *Journal of Virology* **67**, 3126–3133.

Vzorov, A. N. & Compans, R. W. (2000). Effect of the cytoplasmic domain of the simian immunodeficiency virus envelope protein on incorporation of heterologous envelope proteins and sensitivity to neutralization. *Journal of Virology* **74**, 8219–8225.

Webster, R. G. & Laver, W. G. (1967). Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus. *Journal of Immunology* **99**, 49–55.

Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J. & Wiley, D. C. (1997). Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**, 426–430.

Wetz, K. (1993). Attachment of neutralizing antibodies stabilizes the capsid of poliovirus against uncoating. *Virology* **192**, 465–472.

Wetz, K., Willingmann, P., Zeichhardt, H. & Habermehl, K. O. (1986). Neutralization of poliovirus by polyclonal antibodies requires binding of a single IgG molecule per virion. *Archives of Virology* **91**, 207–220.

Wilson, C., Reitz, M. S., Jr, Aldrich, K., Klasse, P. J., Blomberg, J., Gallo, R. C. & Robert-Guroff, M. (1990). The site of an immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 does not constitute the neutralization epitope. *Journal of Virology* **64**, 3240–3248.

Wohlfart, C. (1988). Neutralization of adenoviruses: kinetics, stoichiometry, and mechanism. *Journal of Virology* **62**, 2321–2328.

Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A. A., Desjardin, E., Newman, W., Gerard, C. & Sodroski, J. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384**, 179–183.

WuDunn, D. & Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *Journal of Virology* **63**, 52–58.

Zebedee, S. L. & Lamb, R. A. (1988). Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *Journal of Virology* **62**, 2762–2772.

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