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The Antiviral Activity of Antibodies *in Vitro* and *in Vivo*

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I. Introduction

Antibodies can display antiviral activities *in vivo* and *in vitro*. Since experimentation is far easier *in vitro,* researchers have long sought to develop *in vitro* assays that will predict activity *in vivo*. Furthermore, successful prediction implies that one is studying the mechanisms that are operative *in vivo,* and therefore one can hope to manipulate antibody activity. This could be important in both vaccine design and in passive antibody administration. However, despite a large body of work, there are still major controversies in the area. Many of these spring from fundamentally different views of how antibodies neutralize viruses.

The proposed mechanisms of *in vitro* neutralization range from those requiring binding of a single antibody molecule to virus to those requiring substantially complete antibody coating of virus. The relationship between binding to virus and neutralization has attracted controversy, with some researchers arguing for binding but nonneutralizing antibodies, and others dissenting. The relationship between neutralization *in vitro* and protection *in vivo* has attracted much attention, but still one hears debate about whether neutralization *in vitro* is an absolute requirement for efficacy *in vivo*. Of course, all of these considerations can vary from one set of circumstances (virus, antibody, cells used *in vitro,* animals used *in vivo,* etc.) to another, so one can question whether any general rules can be established.

We believe that some general rules can indeed be established. We begin with the viewpoint that antibodies are not molecules like enzymes that have evolved over long time periods for narrow, highly specialized functions. Rather, they are the products of mutation and selection that are generated afresh in each individual animal as a result of antigen challenge. Each antibody, even directed to the same epitope, has a different sequence and a different detailed mode of binding to antigen. Such molecules are not, for example, likely to trigger common conformational changes in the antigen. They can cross-link antigen molecules, but otherwise their modus operandi is likely to be through their bulk and through the ability of the Fc region, in an array, to trigger effector systems. In other words, in the first instance, we look for antibodies to function antivirally by binding to virus or infected cells and blocking steps in the infection process by nonspecific steric interference or by associating on the virus-infected cell surface and triggering effector functions such as complement and

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antibody-dependent cellular cytotoxicity (ADCC). If "straightforward" explanations for antibody activity fail, then more complex explanations can be considered. It is in this context that we present this review of the literature on the *in vitro* and *in vivo* antiviral activities of antibodies. We have included data from early studies up to the present day, as we believe that some early studies have been misinterpreted, and the misconceptions have been propagated ever since.

In vivo, it is conventional to distinguish phenomenologically between two types of antibody antiviral activity. One is the ability of antibody to protect against infection when it is present before or immediately following infection. The other is the ability of antibody to interfere with an established infection. There is good reason to make this distinction because there is strong evidence that antibody is effective against many different viruses in a protective role, but there is more ambiguity about a role in ongoing infection. The distinction may have its origins, at least in part, in the activity of antibody against propagation of infection by free virus compared to its activity against propagation via cell-to-cell spread. Evidence for a number of viruses *in vitro* indicates that lower antibody concentrations are required to inhibit infection propagated by free virus than are required to inhibit infection propagated by cell-to-cell spread.

In vitro, antiviral activity can be separated into activity against virions and activity against infected cells. The activity against virions most often considered is neutralization, which can be defined as

> the loss of infectivity which ensues when antibody molecule(s) bind to a virus particle, and usually occurs without the involvement of any other agency. As such this is an unusual activity of antibody paralleled only by the inhibition of toxins and enzymes
>
> Dimmock, 1995

Antibody-dependent complement-mediated virolysis is also an activity directed against free virions. Antibodies can also bind to viral products on infected cells to trigger effector functions, including activation of complement and ADCC. Antibody-mediated phagocytosis can be directed against free virions as well as some infected cells. In addition, the binding of antibody to infected cells has been proposed to inhibit intracellular viral replication in certain cases. We begin by considering the *in vitro* antiviral activities of antibody.

II. Mechanisms of Neutralization

A. Introduction

Neutralization of viruses by antibody has been extensively studied during the last century, and many mechanisms for neutralization have been proposed (reviewed in Burnet *et al.,* 1937; Daniels, 1975; Della-Porte and Westaway, 1977; Dimmock, 1993; Fazekas de St.Groth, 1962; Parren *et al.,* 1999). These mechanisms include aggregation, inhibition of viral entry by inhibition of

attachment and inhibition of fusion with the target cell, as well as post entry mechanisms, such as interference with primary and secondary uncoating of the genetic information of the virus (e.g., Dimmock, 1993). It has been suggested that some antibodies may neutralize by acting via several mechanisms simultaneously or sequentially (Dimmock, 1993).

Kinetic studies of virus neutralization have been interpreted to indicate that neutralization often follows single- or few-hit kinetics in which the binding of a single or a few antibodies triggers an event leading to virion inactivation (Dimmock, 1993; Dulbecco *et al.,* 1956; Mandel, 1976; McLain and Dimmock, 1994). To explain single-hit neutralization of viruses in cases where a number of antibody molecules are bound to a virus particle at the neutralization event [e.g., 4 antibody molecules per virion to poliovirus or 70 per virion in the case of influenza A (see below, and Icenogle *et al.,* 1983; Taylor *et al.,* 1987)], antibody-induced conformational changes of the viral capsid (Mandel, 1976) or the existence of antigenically identical "neutralization-relevant" and "neutralization-irrelevant" antibody binding sites on the virion surface have been proposed (Icenogle *et al.,* 1983; Taylor *et al.,* 1987). The myriad of proposed neutralization mechanisms, however, are somewhat difficult to reconcile with the nature of antibody generation and selection. Antibodies are superb binding molecules selected for high-affinity binding rather than for their ability to induce critical conformational changes in viral capsid proteins or envelope spikes.

In this review, we propose a much more simplistic and unifying mechanism for neutralization. We suggest that neutralizing antibodies generally act by coating the surface of a pathogen, and that neutralized pathogens represent entities on which this coating has reached a critical density. This antibody coat then prevents the pathogen from interacting properly with the target cell, thereby interfering with the initiation of a productive infection. Differential effects of the antibody coat on distinct viruses, such as inhibition of attachment, viral entry, or apparent post entry effects, may be due to the epitope being recognized, but also may in part be explained by differences in virus biology rather than by the direct induction of specific events by the antibody. The mechanism suggests that so-called "neutralizing" and "nonneutralizing" epitopes do not exist as distinct entities on the viral surface, as has been suggested in the past (e.g., Dimmock, 1993). Neutralizing epitopes generally represent antibody-accessible structures on the virion surface which are present in a valence high enough that a critical antibody density, as will be calculated below, can be achieved. Low-density epitopes do not represent true nonneutralizing epitopes, as critical density could be achieved by a combination of two or more antibodies against several distinct low-density epitopes. Neo-epitopes which may exist only transiently on virions, for example during virus–target cell fusion, do not necessarily form an exception to the model discussed above. In our view, antibodies against such epitopes will only be neutralizing if the neo-epitope is accessible to antibody at some point during the virus infection process so that effective coating can occur.

Neutralizing antibodies against such cryptic epitopes, however, appear to be relatively rare.

B. Antibody Neutralization and Binding to Virus

Neutralization of a virus by antibody requires that the antibody bind to the coat of the virus. Therefore, antibody binding to virions may be a useful indicator of *in vivo* activity. In reality, the measurement of antibody binding to virions is often technically demanding. Furthermore, for many virus preparations, infectious particle:total particle ratios are low, introducing an element of uncertainty with regard to envelope homogeneity. Therefore, measurement of binding to envelope at the surface of infected cells has often been preferred. It should be noted, though, that some viruses express nonstructural or modified viral proteins on the infected-cell surface but not on virions. Such (non-neutralizing) antibodies are ineffective against free virus, but may be able to protect against viral infection *in vivo* by mediating the destruction of infected cells. This will be discussed in more detail below.

For human immunodeficiency virus type 1 (HIV-1), a very good correlation has been described between neutralization and binding to envelope spikes on the surface of infected cells (Parren *et al.,* 1998; Roben *et al.,* 1994; Sattentau and Moore, 1995). However, problems can arise with the measurement of binding to envelope on cell surfaces. It is important to minimize these problems as much as possible by proper experimental design. First, envelope spikes such as those of HIV-1 can shed protein, providing opportunities for observation of antibodies that apparently bind well to the infected cell surface but do not bind to functional envelope spikes. Some antibodies against the HIV-1 transmembrane (TM) unit gp41 probably fall in to this category, as they may bind to inactivated envelope spikes in which gp41 is exposed, following shedding of the surface unit (SU) gp120. A second type of artifact in HIV-1 is provided by antibody binding to shed gp120 in interaction with the primary HIV-1 receptor CD4; antibodies apparently bind to the cells, but this does not reflect binding to envelope spikes. This is significant, as many nonneutralizing epitopes which are not accessible on the oligomeric HIV-1 envelope spike are exposed on monomeric gp120 (Moore *et al.,* 1995; Parren *et al.,* 1999). A third type of artifact may arise if envelope is overexpressed, for example, by expression of recombinant envelope glycoprotein under the control of a strong promotor. Thus it seems clear that the unprocessed HIV-1 envelope precursor glycoprotein gp160 can be expressed at cell surfaces under certain conditions (e.g., Dubay *et al.,* 1995; Karlsson *et al.,* 1996). Since many antibodies that do not bind to envelope spikes bind well to unprocessed gp160, there is again an opportunity for erroneous conclusions (Parren *et al.,* 1997b).

While there is general agreement that only anti-envelope antibodies that bind to the envelope spike on the virion will be neutralizing, there is considerably

less agreement about whether all antibodies that bind to the envelope spike will neutralize virus, that is, are there antibodies that bind well to envelope spikes but do not neutralize, virus? The answer to the question has a number of important ramifications. If many antibodies that bind to envelope spikes do not neutralize, then binding is not necessarily a good indicator for likely antiviral efficacy. Further, there are implications for vaccine design. If all antibodies that bind also neutralize, then the envelope spike has the antigenic properties of an ideal vaccine candidate. On the other hand, if the envelope spike can induce nonneutralizing antibodies, it may not be an optimal antigen. In particular, the induction of nonneutralizing antibodies that can bind to envelope spikes and inhibit the binding of neutralizing antibodies would be undesirable.

Our opinion on the issue of nonneutralizing but binding antibodies has been presented recently (Burton *et al.,* 2000). We summarize here. We argue that overwhelmingly there is a good correlation between neutralization and virion binding with few exceptions. Our own experience in studies on human monoclonal antibodies (mAbs) to HIV-1, respiratory syncytial virus (RSV), and Ebola virus, has been that there is an excellent correlation between binding to envelope spikes and neutralization, which in these studies was measured as binding to infected cells. We have generally seen a close correlation between half-maximal antibody binding and antibody concentration required to give 50% neutralization, suggesting that neutralization is directly related to occupancy of sites on the virion. For HIV-1, neutralization is incremental with increasing antibody occupancy, irrespective of the epitope recognized, leading to increased inhibition of infectivity (Parren *et al.,* 1998; Schønning *et al.,* 1999). This issue is discussed in much greater detail below.

Irrespective of our observations, there is a strong tradition in virology of "binding (i.e., to the virion) but nonneutralizing" antibodies. Early studies described a "nonneutralizable" fraction of virus which persisted even at high antibody concentrations. Addition of anti-antibody could reduce infectivity of this fraction. It seems that virus aggregation may have been partly responsible for this phenomenon (Wallis and Melnick, 1967). This will be discussed in more detail below. We also note that nonneutralizable fractions have been described for hepatitis A and hepatitis C as a result of virus association with lipids or lipoproteins (Lemon and Binn, 1985; Prince, 1994).

Some descriptions of binding, nonneutralizing antibodies arose because of a failure to appreciate that antibodies that bind to isolated envelope molecules do not necessarily, and very often do not, bind to envelope spikes. A classic example is HIV-1, where many antibodies have been described which bind with high affinity to monomeric gp120 or unprocessed gp160; very few of these, however, show substantial affinity for envelope spikes (reviewed in Parren *et al.,* 1999).

Antibody-mediated enhancement of infection is a phenomenon that may support the existence of nonneutralizing but binding antibodies since the antibodies

involved must bind to virions. However, a key observation here is that enhancement, when described, appears to occur for neutralizing antibodies at subneutralizing concentrations (e.g., Halstead, 1982; Morens *et al.,* 1987). We favor the view that antibody enhancement is an effect that can arise under conditions of low-level antibody coating of virions rather than an indication of antibodies with the ability to bind well to virions but not neutralize. This is discussed in detail below. The phenomenon does suggest, however, a potential problem for some vaccines that induce low levels of neutralizing antibodies

A clear example of good evidence for an antibody which binds well to virion envelope spikes without effecting neutralization, at concentrations where it would be expected to completely coat the virion, is provided from studies on rabies virus, as discussed later (Flamand *et al.,* 1993). However, here it appears to be the rabies mutant virus rather than the antibody which has unusual properties.

C. Early Studies on Antibody Neutralization

At the end of the nineteeth century, Willem Beijerinck made an important conceptional advance with the recognition that viruses, which he termed the "contagium vivum fluidum," represent infectious entities distinct from bacteria (Beijerinck, 1898; Van Kammen, 1999). Great advances in understanding of the inactivation of viruses by antibody were then made in the early twentieth century. In a prominent article, Burnet and colleagues reviewed the state-of-the-art of antibody neutralization in 1937 (Burnet *et al.,* 1937). They argued that neutralization studies of bacteriophage, plant, and animal viruses showed that generally (i) neutralization was reversible, and (ii) more than one antibody was required for neutralization. Based on studies with a number of animal viruses including vaccinia and influenza, they summarized: "virus and antibody combined reversibly, and that the infectivity or otherwise of a given virus particle was determined by the amount of antibody united to it at the time of effective contact with the susceptible cell." Reversibility indicated that the "union of antibody has no intrinsic inactivating effect on the virus." The conclusions that neutralization is reversible and requires the binding of multiple antibody molecules to the virion have since been disputed (and rediscovered) in many later publications. In particular, the observation of single-hit kinetics in a number of experimental systems has been interpreted as evidence for neutralization by the binding of a single antibody to a critical site on the virion.

In 1956, Jerne and Avegno showed that inactivation of T4 bacteriophage followed first-order kinetics, and inactivation may be the result of a single event. This was plausible, as neutralizing antibodies bind to the tail-plate of the phage, an essential component in the infective process, and the width of the tail-plate is similar to the length of an antibody molecule (Jerne and Avegno, 1956). An influential study which sharply contradicted the conclusions by Burnet *et al.* (1937) was published by Dulbecco and colleagues also in 1956, who analyzed

neutralization of Western equine encephalitis virus (WEE) and poliovirus by specific antisera (Dulbecco *et al.,* 1956). It was found that curves describing the logarithm of virus survival as a function of time generally did not display an initial shoulder or lag time, which was interpreted as neutralization following first-order kinetics. After an initial time period, however, the slope of the curves decreased, eventually becoming horizontal, which indicated the existence of a persistent and apparently neutralization-resistant fraction of virus. Dissociation of poliovirus-antibody complexes could not be observed, and there was only a very slight dissociation of WEE-antibody complexes. It was concluded that the attachment of a single neutralizing antibody molecule to a critical site is sufficient to inactivate a virus particle and that neutralization is irreversible (Dulbecco *et al.,* 1956).

However, not all results in the manuscript by Dulbecco *et al*. could be explained by the neutralization model they developed. First, a clear lag time of WEE neutralization was observed when kinetic neutralization experiments were performed at temperatures lower than 37°C. Even at 37°C, Dulbecco *et al*. noted that a small lag time may exist, although the experimental conditions used may have made detection difficult. Furthermore, multiplicity curves, which describe the logarithm of virus survival as a function of increasing antibody concentration, did not follow theoretical curves based on the model; experimental curves bent upward at high antibody concentration rather than downward. This deviation could not be completely explained by the persistent virus fraction. The authors concluded that different fractions may be present in the challenge stock consisting of virions with a different number of critical sites or unequal distribution of critical sites (Dulbecco *et al.,* 1956).

Krummel and Uhr (1969) recognized that the critical site neutralization models developed often did not fit the data at later time points during the course of neutralization in kinetic experiments. They argued that, for a highly symmetrical virus with a surface array of repeated coat-protein subunits such as the bacteriophage ϕX174 studied, it was likely that multiple antibody molecules were required for neutralization. Kinetic neutralization experiments, in which residual infectivity was assessed using two different methods, indeed indicated that bacteriophage covered with antibody may retain its infectivity. Significantly, they observed an apparent faster neutralization rate if residual infectivity was assessed using a decision tube method rather than the conventional direct plating method. In a kinetic neutralization experiment, antibody and phage are mixed, and samples, drawn at multiple time points, are incubated with bacteria and plated to assess residual infectivity. In the decision tube method, an excess of additional neutralizing antiserum is added a short time after adding the reaction sample to the bacteria. Whereas infectious titers determined with both methods were similar prior to the addition of neutralizing antibody, the decision tube method measured markedly decreased infectious titers compared to the

conventional plating method at time points after the addition of antibody. As neutralization of ϕX174 phage was effectively irreversible under the conditions used, the difference observed could not be explained by the prevention of reactivation of neutralized phage (e.g., by dissociation of antibody–virus complexes). A plausible explanation is that some phage are partially coated with antibody molecules but not neutralized, and these phage infect bacteria at a slower rate than naked phage. A similar delay in the initiation of bacteriophage-induced lysis in neutralization experiments has previously been interpreted to indicate that partially antibody-coated but infectious phage particles exist (reviewed in Burnet *et al.,* 1937). By entering an infectivity function for such phage–antibody complexes into the mathematical model developed by Krummel and Uhr (1969), the model better matched their data. This clearly indicated that the observed reaction rates of neutralizing antibody with bacteriophage were not first order, even under conditions of antibody excess (Krummel and Uhr, 1969).

D. The Case of Picornaviruses

Several mechanisms have been described for the neutralization of picornaviruses, including aggregation, virion stabilization, inhibition of virus attachment, and induction of conformational changes in the viral capsid (see Smith, 2001, for a recent review). The induction of conformational changes (resulting in a large change in isoelectric point) upon antibody binding is the most prominent, and has prompted the most detailed investigations. Most studies have been carried out with poliovirus and human rhinovirus (HRV).

Neutralization of poliovirus has been studied extensively. Dulbecco *et al*. (1956) argued that neutralization of poliovirus is irreversible and follows first-order kinetics as discussed. Studies performed by Mandel (1961) confirmed that reactivation of virus infectivity after dilution of antibody–virus complexes could not be demonstrated in measurements taken over several months. The apparent irreversibility of the reaction appeared to be due, however, to a very tight binding of antibody to poliovirus rather than the induction of permanent changes in the viral particles, as it was demonstrated that neutralizing rabbit antibody could be eluted from poliovirions at low pH accompanied by quantitative reactivation of virus activity (Mandel, 1961). Mandel (1976) found that the capsid protein of poliovirus may exist in two conformational states, characterized by distinct isoelectric points: state A, with an isoelectric point of about 7, and state B, with an isoelectric point of about 4.5. Neutralizing antibody was found to stabilize the virion in state B. Mandel proposed that binding of neutralizing antibody locked the viral capsid in a noninfectious conformation by rendering it resistant to uncoating following its adsorption to the target cell. Single-hit kinetics in poliovirus neutralization could be explained by the induction of a generalized molecular rearrangement in the viral capsid after binding of a single antibody to a critical site on the capsid molecule. The mechanism is feasible, as the poliovirus capsid

is a closely packed structure making conformational cooperativity among capsid subunits a possibility (Mandel, 1976). However, the relationship between the conformational state and infectivity was not complete, as infectious virus in the B state could be detected, particularly at low antibody multiplicity (even after subtracting the infectivity assigned to the small fraction of virus naturally occurring in the B state) (Mandel, 1976). A correlation between binding of neutralizing antibody to poliovirus and a shift in isoelectric point has generally held up in studies with monoclonal antibodies (Emini *et al.,* 1983a), although exceptions have been noted (Brioen *et al.,* 1985; Emini *et al.,* 1983a).

Changes in isoelectric point following the binding of neutralizing antibody have also been described for another picornavirus, namely HRV(Colonno *et al.,* 1989). However, in this case it is particularly clear that the induced conformational changes do not play a major role in picornavirus neutralization (reviewed in Hewat and Blaas, 2001; Smith, 2001). The most convincing data come from cryo-electron microscopy (cryo-EM) and X-ray crystallography of virus–antibody complexes which show that rhinovirus can be neutralized without the induction of large conformational changes in the virion capsid (Hewat and Blaas, 1996; Smith, 2001; Smith *et al.,* 1993, 1996). Furthermore, as the change in isoelectric point has been observed following binding of a large number of different antibodies against a range of epitopes on rhinovirus, it appears unlikely that all these antibodies would induce a common conformational change required for neutralization. The most likely hypothesis is that the changes in isoelectric point are the result of changes occurring in exposed highly flexible portions of the capsid proteins rather than structural rearrangement of the viral capsid (Che *et al.,* 1998; Smith, 2001). Although, being the result of the binding of neutralizing antibody, these changes are probably mostly irrelevant to the mechanism of neutralization itself.

The antibody activity most likely to affect HRV infectivity appears to be inhibition of virus attachment. The strongly neutralizing mAb 17-IA binds to an epitope located on a loop between the B and C strands of the capsid protein VP1 β-barrel and situated close to the canyon which harbors the binding site for the cellular HRV receptor ICAM-1 (reviewed in Smith, 2001). mAb 17-IA neutralizes by inhibition of attachment, and it has been demonstrated that attachment of 6–7 antibody molecules per HRV particle is required to reduce attachment by 63% (Smith *et al.,* 1993). Significantly, Colonno *et al.* (1989) have shown that Fab fragments of antibodies against all four neutralizing epitopes on HRV inhibit attachment, some of which are located more-distal from the HRV receptor binding site. A linear correlation was found when inhibition of attachment was plotted as a function of antibody concentration. Furthermore, the rank order of neutralization potency followed the rank order of inhibition of attachment. The studies on HRV clearly demonstrate that the binding of more than one antibody molecule is required for neutralization, and suggest a role

for antibody occupancy of binding sites on the virus, as will be discussed in the following sections.

Further studies on the neutralization of poliovirus suggest that the neutralization of this picornavirus also requires the attachment of multiple antibody molecules. Particular insight into the relationship between antibody bound to poliovirus and neutralization was provided by Philipson and colleagues (1966) who developed an aqueous polymer phase system to study the interaction between neutralizing antibody and poliovirus. It was found that virions complexed with antibody could be separated from free virions by adding a polymer to the mixture, followed by a simple phase-separation technique. The phase system was more sensitive in detecting antibody binding to the virion compared to conventional neutralization tests, clearly indicating that neutralizing antibody was bound to the virions before neutralization could be detected. Interestingly, binding titers in the phase system method were 2–4 times greater than the serum neutralization titers against poliovirus (Philipson *et al.,* 1966). The significance of this number will become clear shortly. Additional studies by Philipson on the interaction of antibody with poliovirus using countercurrent distribution, furthermore, showed that at low antibody input, virion–antibody complexes separated from free virus retained infectivity (Philipson, 1966).

The neutralizing antibody dose is defined as antibody concentration at which virus infectivity is reduced to 37% (1/e) of the infectivity of the nonneutralized virus stock. Icenogle *et al.* (1983) determined the neutralizing dose for poliovirus in a study using radiolabeled virus and antibody, and demonstrated that the binding of an average of 4–5 antibody molecules to a poliovirus particle resulted in neutralization. Two neutralization models were proposed to explain these results in the context of first-order kinetics: a critical site hypothesis, in which binding to one out of four sites on the virus leads to neutralization, and a stepwise model, in which binding to each site reduces infectivity by 3/4. Both models, however, are in conflict with the studies by Philipson *et al.* (1966), discussed above, which demonstrated that antibody binding to virions can be detected prior to neutralization. Significantly, the observation that an occupancy of 4–5 antibody molecules per virion is required for neutralization is consistent with the observed differences in binding and neutralization titers in the Philipson studies. The most straightforward conclusion is that the single-hit hypothesis of poliovirus neutralization is not correct, and that the mechanism of neutralization of poliovirus is similar to that of HRV.

The overall architecture of poliovirus and HRV are similar, and both viruses have similar proportions consisting of a 30-nm-diameter capsid surrounding the positive-strand RNA genome. The three-dimensional structure of poliovirus in complex with a soluble form of its receptor (CD155) has recently been solved (Belnap *et al.,* 2000; He *et al.,* 2000). The poliovirus receptor CD155 has a footprint similar to that observed for the rhinovirus receptor ICAM-1 bound to

HRV. Both bind to the canyon, a narrow surface depression on the viral surface. It may therefore not be completely surprising that the stoichiometrical analyses discussed above yield similar results for HRV and poliovirus. The data suggest that the attachment of 4–7 antibody molecules is required for the neutralization of picornaviruses of poliovirus/HRV dimensions.

Studies by Horaud and colleagues (Blondel *et al.,* 1986; Crainic *et al.,* 1983; Diamond *et al.,* 1985) have suggested that antibodies may bind to poliovirus without neutralization. This conclusion was made on the basis of studies using neutralizing antibodies which were analyzed for their activity (neutralization and binding) against a range of antigenic poliovirus variants (distinct serotypes and escape mutants). For example, some serotypes appeared to be bound by antibody in an immunoprecipitation assay but were not neutralized. The studies, however, made no distinction between strong and weak binding. Mutations on poliovirus envelope in or near the antibody binding site may result in decreased antibody affinity for the poliovirus mutants in question, resulting in a level of occupancy too low to neutralize the virus (for example, as shown by the studies discussed above). This interpretation is supported by the observation that addition of anti-antibody in the studies above did result in neutralization of the mutant viruses (Blondel *et al.,* 1986), presumably because anti-antibody effectively increases affinity (avidity) and raises occupancy to the level required for neutralization (see below).

E. Single-Hit Versus Multi-Hit Kinetics

1. Overview

Single-hit models are based on the observation that infectivity starts to decrease immediately following addition of antibody to virus. The absence of a detectable lag phase, i.e., a period in which infectivity remains unaffected following antibody addition, has often been taken as supporting evidence for the single-hit model (Dulbecco *et al.,* 1956; Icenogle *et al.,* 1983; Mandel, 1976; McLain and Dimmock, 1994; Taylor *et al.,* 1987). It may be argued, however, that reactions of antibodies of nanomolar affinities for their cognate antigen occur rapidly (e.g., Scicluna and McCullough, 1999), and as samples are being drawn in kinetic studies at intervals of minutes or greater, it may not be possible to measure the surviving virus fraction fast enough to observe the presence or absence of a lag phase under standard conditions (Della-Porte and Westaway, 1977). Furthermore, lag times in neutralization kinetics have indeed been observed in studies with most viruses when the experiments were performed at lower incubation temperature, more dilute antibody concentration, or with antibodies of lower affinity (Burnet *et al.,* 1937; Dulbecco *et al.,* 1956; Lafferty, 1963a; Philipson, 1966; Taylor *et al.,* 1987). This is difficult to reconcile with the single-hit model, as it requires the assumption that the mechanism of

neutralization changes as temperature, antibody concentration, or affinity are varied.

Klasse and Moore (1996, and references therein) have argued that a first-order neutralization reaction does not give direct proof for neutralization by single-hit kinetics. One antibody molecule neutralizing one virion implies first-order kinetics, but the reverse is not true. Stoichiometrical analyses (see below) suggest that the binding of more than one antibody molecule to a virus particle is usually required for neutralization. An alternative explanation for the absence of a lag phase to the one discussed above, at least for enveloped viruses, may be found in a heterogeneity of spike density. In virus stocks, virions may exist with a low number of active spikes that may only require one or a few hits for neutralization (Klasse and Moore, 1996). HIV-1 is an example where this hypothesis may be applicable, as shedding of gp120 from the virion results in the formation of inactive spikes (Parren *et al.,* 1999; Sattentau *et al.,* 1995).

A plethora of evidence indicates that virus neutralization in general is the result of the attachment of a number of antibody molecules to the infective particle. Many of these arguments have been cogently reviewed by Della-Porta and Westaway (1977). A summary of these arguments, supplemented with evidence for multi-hit neutralization from more recent studies, is presented here. These arguments include:

- the occurrence of a persistent fraction of infectious virus
- the neutralization of infectious virus–antibody complexes (sensitized virus) by addition of anti-antibody
- neutralization of sensitized virus by addition of complement
- retardation of infection of sensitized virus
- the phenomenon of antibody-mediated enhancement of infection
- additive and synergistic effects of antibodies in neutralization

2. The Persistent Fraction of Infectious Virus

The presence of a persistent fraction of nonneutralizable virus in most virus preparations has been the source of much debate. The nonneutralizable virus fraction has been attributed to the presence of low-affinity antibodies in antibody preparations (particularly early immune sera), virus heterogeneity, neutralization-interference of nonneutralizing antibodies, virus association with lipids and lipoproteins, and virus aggregation. In some studies, it has been shown that the infectious titer of the persistent fraction can be further reduced by the addition of anti-antibody, implying that this fraction contains virus particles with some level of antibody coating.

An early explanation of the nonneutralizable virus fraction attributed the phenomenon to association–dissociation equilibria between virus and antibody (Burnet *et al.,* 1937). Indeed, dissociation may play a role when low-affinity

antibody preparations are being used, and dissociation of the virus–antibody complexes occurs before or during the addition of the complexes to the target cell (Jerne and Avegno, 1956; Lafferty, 1963b). With high-affinity antibody preparations, however, this does not seem to be an important factor (Dulbecco *et al.,* 1956). It has also been suggested that a low-affinity antibody or nonneutralizing antibody may bind to the virion, preventing the binding of neutralizing antibody and giving rise to a nonneutralizable fraction. In some cases, however, such a fraction is more easily explained in terms of heterogeneity of the virus preparation. Vaccinia virus and rabbitpox virus preparations, for example, contain a small fraction of extracellular enveloped virus (EEV) in an excess of intracellular mature virus (IMV). The EEV contains an additional wrapping membrane and is relatively resistant to neutralization (Ichihashi, 1996; Vanderplasschen *et al.,* 1997). The wrapping membrane however is easily damaged during manipulations thereby exposing the more-neutralization sensitive IMV particle (Ichihashi, 1996; Vanderplasschen *et al.,* 1997). This mechanism, rather than dissociation of low-affinity or nonneutralizing antibody, may explain experiments that show that the persistent fraction in rabbitpox virus can be neutralized following dilution and washing (Lafferty, 1963a).

In a study on neutralizing antibodies against mouse mammary tumor virus (MMTV), it was suggested that nonneutralizing mAbs against MMTV gp52 could interfere with the binding and neutralizing activity of a neutralizing mAb (Massey and Schochetman, 1981). This would be one of the only examples of such an activity for a monoclonal antibody. The inhibition described, however, is extremely weak and only apparent at high nonneutralizing mAb:neutralizing mAb ratios. On closer examination, the observed blocking, furthermore, is well within the range of the experimental error of the MMTV pseudotype neutralization assay used (Massey and Schochetman, 1981), and is therefore unlikely to be of any significance.

Aggregation of virus may be responsible for many observations of a nonneutralizable virus fraction. Aggregates of virus particles already present in the preparation, or formed by aggregation with antibody, may inhibit access of antibody to virus particles at the inside of a small aggregate protecting it from neutralization. Filtration to remove the aggregate or treatment with anti-antibody to coat the aggregate more extensively can indeed remove the persistent fraction in some instances (Wallis and Melnick, 1967, 1970).

3. Neutralization of Sensitized Virus with Anti-antibody or Complement

Neutralization of sensitized virus by anti-antibody or complement components has been cited as an argument against single-hit kinetics, as it indicates that infectious virus–antibody complexes exist (Della-Porte and Westaway, 1977).

4. Retardation of Infection by Antibody

Burnet *et al.* (1937) noted in their review that infectious antibody–bacteriophage complexes exist, as evidenced by observations in the literature that plaque sizes were reduced in the presence of antibody, which presumably occurred through delayed infection of phages with a low level of antibody attached. Krummel and Uhr (1969) demonstrated reduced infectivity rates for bacteriophages complexed with neutralizing antibody in kinetic neutralization experiments (see above). Westaway (1965) further noted that the rate of penetration of West Nile virus preincubated with neutralizing antibody was delayed, as the virus was susceptible to postattachment neutralization for a longer period of time after adsorption to target cells than nonsensitized virus. These experiments indicate that antibody may combine with virus at an occupancy too low to neutralize the virus but high enough to decrease the rate of entry into the target cell.

5. Antibody-Mediated Enhancement of Infection

Antibody-mediated enhancement of infection is a phenomenon that appears to occur for neutralizing antibodies at subneutralizing concentrations (Halstead, 1982; Hawkes and Lafferty, 1967; Morens *et al.,* 1987). Enhancement is very sensitive to the target cell and is dependent on the types and expression levels of Fc and/or complement receptors on the target cell surface. A classical example is enhancement of dengue virus infection, which is dependent on the interaction between virion-bound antibody and Fc receptors expressed on the target cell. In typical assays, neutralization is observed at relatively high concentrations, whereas enhancement is observed at lower concentrations. Using neutralizing antibodies against dengue virus, Morens *et al.* (1987) indeed showed that the mAb concentration maximizing enhancement was predicted by the neutralization titer. Hawkes and Lafferty (1967) noted that the balance between enhancement and neutralization is dependent on the incubation time of virus and antibody. They observed a shift of the dose response curve as time progressed, and enhancement as well as neutralization occurred at increasingly lower concentrations. Presumably, at early time points, attachment of a small number of antibodies leads to enhancement, and as the reaction progresses, more antibodies attach, eventually leading to neutralization of the virion. This clearly indicates that enhancement and neutralization are two different biological outcomes of the interaction of an antibody with virus at different levels of occupancy.

For HIV-1, both Fc receptor-mediated and complement-mediated antibody-dependent enhancement of infection have been described (Homsy *et al.,* 1989; Lund *et al.,* 1995; Mascola *et al.,* 1993; Robinson *et al.,* 1988; Schutten *et al.,* 1995; Takeda *et al.,* 1988). In Fc receptor-mediated enhancement, Fc receptor-mediated endocytosis of virion–antibody complexes may lead to the internalization of virus and infection. A plausible alternative is that binding to Fc receptors

stabilizes the interaction of virion and target-cell, permitting interaction of the envelope spike and virus receptor at low antibody coating of the virion (Connor *et al.,* 1991). At higher coating, this interaction may be inhibited. Complement receptor 2 (CD21) may mediate enhancement of HIV-1 infection via several proposed mechanisms, either by acting as a receptor for HIV-1 opsonized with antibody and complement or by increasing virus binding to the cell due to an interaction of CD21 with opsonized virus (Boyer *et al.,* 1992; Lund *et al.,* 1995). In addition, virion-bound C1q may directly interact with receptors on the host cell, leading to enhancement of infection (Prohaszka *et al.,* 1997). In some cases, such as HIV-1, the enhancement does not require the Fc part of the antibody molecule (Sullivan *et al.,* 1998b). It is suggested that the low-level coating may trigger conformational changes in the envelope that, for example, favor fusion between the virion and target cell, or such coating may nonspecifically reduce repulsion between the virion and target cell surface. A similar mechanism of antibody-mediated enhancement of infection has been described for Sindbis virus (Flynn *et al.,* 1988).

The phenomenon of enhancement indicates that antibodies bind to virions at subneutralizing concentrations and is therefore a strong argument for the importance of antibody occupancy in virus neutralization and neutralization following multi-hit kinetics. High levels of occupancy lead to neutralization, whereas, in the presence of permissive target cells, low levels of occupancy may lead to enhancement of infection.

6. Additive and Synergistic Effects in Neutralization

The presence of additive and synergistic effects in neutralization is indicative of a role of antibody occupancy in neutralization of viruses and provides an argument for multi-hit neutralization. Phenotypic mixing experiments, in particular, are informative in this respect. It has been shown that cells dually infected with certain viruses may yield phenotypically mixed virions. These are relatively poorly neutralized by single specificity antisera against each of the parent viruses, but are more effectively neutralized by mixed neutralizing antisera. Cells dually infected with the myxovirus fowl plaque virus (FPV) and the rhabdovirus vesicular stomatitis virus (VSV) produced a virus progeny of which about 20% was composed of virus particles with mixed envelopes. Neutralization of virions carrying mixed envelopes was poor to nonexistent in neutralization assays with anti-VSV and anti-FPV alone. However, strong neutralization occurred when the assay was carried out with a mixture of the two sera (Zavada and Rosenbergova, 1972). Similar observations were made with phenotypically mixed virions of VSV and Sendai virus (Kimura, 1973) and VSV and measles virus (Wild *et al.,* 1975–1976).

Additive effects are also observed for antibodies binding to the paramyxovirus Newcastle disease virus (NDV). Binding of single mAbs against the NDV

hemagglutinin-neuraminidase (HN) glycoprotein leaves a large persistent fraction of nonneutralized virus (Iorio and Bratt, 1983). The persistent fraction appeared to be bound by neutralizing mAb, as it was almost completely neutralized by the addition of anti-antibody (Iorio and Bratt, 1984). It did not seem to contain large aggregates of virus or genotypically distinct viruses (Iorio and Bratt, 1984). Interestingly, the persistent fraction could be neutralized when antibodies against several epitopes on the HN glycoprotein were combined and, in addition, a level of neutralization comparable to a polyclonal immune serum could only be obtained by combining antibodies against four distinct epitopes on NDV (Iorio and Bratt, 1983, 1984). These observations are consistent with some heterogeneity of the NDV HN glycoprotein which may exist in different conformations on the virion surface. Neutralization of La Crosse virus to the level of a polyclonal antibody could similarly only be obtained with combinations of two monoclonal antibodies against the G1 glycoprotein (Kingsford, 1984). These studies suggest a role for antibody occupancy of viral surface binding sites in neutralization of NDV and La Crosse virus.

F. Neutralization of Virions by Coating with Complement

Of interest is that coating virions by early components of the complement system (C1-4) may enhance neutralization without inducing virolysis, which will be discussed below (reviewed by Oldstone, 1975). For HIV-1, it has been shown that the infectivity of plasma virions may be reduced by an *in vivo* deposition of C3 on the virion surface (Sullivan *et al.,* 1998a). Presumably, the complement coat interferes with virus interaction with the target cell, thereby contributing to neutralization (Parren *et al.,* 1999).

G. The Case of Adenovirus

Adenovirus is an icosahedral capsid with characteristic long, thin fibers protruding from the 12 vertices. A knob at the end of each fiber serves as the primary receptor attachment site. The penton base protein, located at the base of the fiber, binds to αv integrins via an RGD motif (reviewed by Stewart and Nemerow, 1997). An unusual observation is that an antibody directed against the RGD motif neutralizes adenovirus infection as a Fab fragment but not as a whole IgG molecule (Stewart *et al.,* 1997). Cryo-EM of adenovirus complexed with the Fab indicates that the Fab may occupy all five RGD epitopes on the penton base complex. The whole IgG molecule was shown to bind soluble penton base with a stoichiometry of 2.8, indicating that, per penton base, two IgG molecules may bind bivalently and one monovalently. In the intact virus particle, however, the central fiber presumably provides a source of steric hindrance, preventing the IgG molecules but not the Fab fragments from binding to all five sites on the penton base (Stewart *et al.,* 1997). Neutralization of adenovirus by

an antibody against the penton base protein therefore requires occupancy of all five integrin binding sites.

H. The Case of Rabies Virus

A number of elegant and detailed studies have been carried out by Flamand and colleagues on antibody neutralization of rabies virus. These studies support the general notion that neutralization is the result of coating the virion with antibody molecules (Flamand *et al.,* 1993; Raux *et al.,* 1995). The number of antibody molecules bound per rabies virus particle was determined carefully and correlated with the magnitude of neutralization achieved. It was found that infectivity was completely preserved with fewer than 130 IgG or 30 IgM molecules bound per virion (Flamand *et al.,* 1993). The neutralizing dose, the dose at which infectivity is decreased to 37% (1/e), was between 130 and 320 IgG (40 to 50 IgM) molecules bound per virion depending on the antibody tested. There is a marked threshold effect with a two- to three-fold increase in the number of antibody molecules close to the threshold, leading to a drop in infectious titer of about 1 to 2 logs (Flamand *et al.,* 1993).

Three mAbs were identified which bound to the virus without neutralization. Two of these were subsequently shown to bind to a minor population of envelope spikes in an altered acidic conformation of envelope (Raux *et al.,* 1995). Coating of the virion via this altered spike was too incomplete to permit neutralization. If the virion was maintained at lower pH to convert most spikes to the acidic form, then more antibody bound, and the virus was neutralized. One mAb, which effectively neutralized wild-type virus, appeared to saturate the envelope spikes of a neutralization escape variant (1080 molecules of IgG per virion) but did not neutralize it. The antibody-coated virus was still able to attach to target cells. The authors considered it unlikely that this attachment was occurring through envelope, because of the thickness of the antibody shell coating the virus (Flamand *et al.,* 1993). This case appears to constitute a bona fide example of virion coating by antibody without neutralization. Although it is the rabies mutant virus rather than the antibody that seems be an exception to the rule here, it would be very interesting to elucidate how this mutant virus can escape neutralization while being completely coated with antibody.

Overall, the studies by Flamand *et al*. (1993) strongly suggest that there is a correlation between antibody occupancy and neutralization of rabies virus. The experiments show that neutralization occurs at a mean critical coating density of about 225 IgG or 45 IgM molecules bound per virion.

I. The Case of HIV-1

We have investigated the relative importance of binding site occupancy and epitope specificity in antibody neutralization of HIV-1 using an extensive panel of neutralizing mAbs against HIV-1 (Parren *et al.,* 1998). Neutralization was studied

using T-cell line adapted (TCLA) HIV-1 isolates. The strategy adopted was to compare the binding of a number of antibodies to different gp120 epitopes, presented in the form of functional oligomeric gp120 on infected cells, with their capacity to neutralize the corresponding virus. A concentration of mAb yielding half-maximal binding (K_{50}) and a neutralization titer (ID_{50}) of similar magnitude would be consistent with antibody occupancy of virion binding sites playing a major role in HIV-1 neutralization. Similar K_{50}/ID_{50} ratios for different epitopes would suggest that neutralization is independent of the epitope recognized. Indeed, an excellent correlation ($r = 0.882$; $P < 0.0001$) between neutralization (ID_{50}) and binding to oligomeric gp120 (K_{50}) was observed. Furthermore, the K_{50}/ID_{50} ratios fell, in general, within a relatively narrow range for antibodies to different neutralization epitopes, suggesting that occupancy of binding sites on HIV-1 virions is the major factor in determining neutralization, irrespective of epitope recognized (Parren *et al.,* 1998). This is illustrated in Fig. 1 in which the K_{50}/ID_{50} ratios for a panel of mAbs against five distinct neutralizing epitopes on TCLA HIV-1 isolates are plotted.

We proposed a model in which neutralization is effected by coating of the virion with antibody. For attachment of the virus to the target cell to occur and infection to initiate, it is presumed that multiple contacts in a localized area between gp120 and the primary HIV-1 receptor CD4 must be established. Coating of the virus above a critical density would interrupt this process and

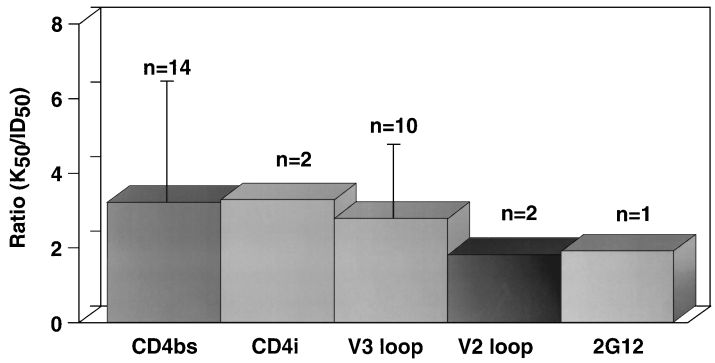

FIG. 1. Neutralization of HIV-1 is determined by antibody occupancy irrespective of epitope recognized. The antibody concentration yielding half-maximal binding (K_{50}) and neutralization titer (ID_{50}) and K_{50}/ID_{50} ratios were determined for a number of neutralizing mAbs against the CD4 binding site (CD4bs), CD4-induced epitope (CD4i), V2 loop, V3 loop, and 2G12 epitopes on gp120 in binding and neutralization assays with HIV-1_{LAI} and HIV-1_{MN} (Parren *et al.,* 1998). Similar K_{50}/ID_{50} ratios for different epitopes suggests that neutralization is independent of the epitope recognized. This suggests that occupancy of binding sites rather than epitope recognized is the major determining factor in neutralization.

thereby inhibit infection (Parren *et al.,* 1998 and reviewed in Parren *et al.,* 1999).

Schønning *et al*. (1999) analyzed the stoichiometry of HIV-1 neutralization in an elegant study by use of virions carrying mixed envelope spikes on their surface. Cells were transfected with an HIV-1 clone (A308), which is potently neutralized by a mAb against the V3 loop on gp120, and a mutant of this clone, T321, which resists neutralization by this antibody by a loss-of-binding mutation. The proportion of antigen binding sites on the virion could now be titrated by transfecting plasmids encoding the two clones in different ratios. Neutralization of the mixed envelope virions was performed with a high concentration of neutralizing antibody (500 × the ID_{50} of the neutralization-sensitive clone), so that all available binding sites would be occupied. Neutralization by virion aggregation did not appear to play a significant role in inactivation. The experiment showed that, as the number of binding sites (and therefore the number of bound antibody molecules) increased, viruses become susceptible to neutralization. Neutralization of 63% of the virions occurs at a level of about 35% occupancy, a level which is consistent with stoichiometric arguments presented below (see Fig. 3). The increase in neutralization is incremental, indicating a multi-hit rather than a single-hit model (Schønning *et al.,* 1999).

It has been shown that average, freshly budded HIV-1 virions carry approximately 72 envelope spikes (216 gp120 molecules) on their surface (Gelderblom, 1991). From the analyses above it can be predicted that the attachment of approximately 70 IgG molecules is required for the neutralization of such HIV-1 virions, which would be equivalent to about one IgG molecule/spike.

Klasse and Moore (1996) developed a quantitative model of HIV-1 neutralization to explain the large difference in neutralization sensitivity between TCLA strains and primary isolates of HIV-1 (see Moore and Ho, 1995, for reviews of HIV-1 neutralization). The model predicts that neutralization of HIV-1 is due to the reduction of the number of active envelope spikes below a minimum required for the induction of a productive infection. Factors contributing to differences in neutralization sensitivity of different virus/antibody combinations are binding constants of neutralizing antibodies to the envelope spike, the number of spikes on the surface, and the minimum number of spikes required for attachment and fusion (Klasse and Moore, 1996). Possible differences in spike density between neutralization-sensitive TCLA strains of HIV-1 (low active spike densities) and neutralization-resistant primary isolates (high active spike densities) are quoted as an important factor in neutralization resistance (Klasse and Moore, 1996). Differences in spike densities between TCLA strains and primary isolates, however, are controversial (Karlsson *et al.,* 1996; Sullivan *et al.,* 1995). Alternatively, the resistance may be due to the poor accessibility of epitopes on the primary isolate envelope spike (Parren *et al.,* 1999).

J. Stoichiometry of Neutralization: A Model for Neutralization Based on Antibody Coating of Virion Particles

The stoichiometry of antibody binding at neutralization has been determined for a number of viruses. We briefly summarize the relevant studies focusing on those where stoichiometry was determined by a direct and quantitative method. Methods that determine the amount of antibody bound using purified radiolabeled virion–antibody complexes (e.g., Taylor *et al.,* 1987), or quantitative sodium dodecyl sulfate–polycrylamide gel electrophoresis (SDS-PAGE) (e.g., Flamand *et al.,* 1993), for example, may give reliable data. Methods in which the stoichiometry is extrapolated from kinetic analysis may be inaccurate because of uncertainties with respect to the mathematical models used, particularly in early studies.

Rappaport (1970) determined that an average of four antibody molecules bound per neutralized MS2 bacteriophage represented a neutralizing dose. Studies by Collono and Smith and their colleagues have indicated that HRV is neutralized by inhibition of attachment, and that the binding of 6–7 antibody molecules per HRV virion is required to reduce HRV attachment by 63% (Colonno *et al.,* 1989; Smith *et al.,* 1993). Icenogle *et al.* (1983) determined that neutralization of poliovirus requires the attachment of 4–5 antibody molecules, as discussed above. The picornaviruses poliovirus and HRV therefore require the attachment of an average of 5–6 antibody molecules for neutralization. A study which assessed the number of IgG molecules necessary to neutralize influenza virus showed for two different mAbs (HC2 and HC10) that approximately 70 molecules were required (Taylor *et al.,* 1987).

Studies by Flamand and colleagues on rabies virus have shown that an average occupancy of 225 IgG molecules per virion is necessary for neutralization (see Flamand *et al.,* 1993; Raux *et al.,* 1995). Studies on bovine papillomavirus (BPV) determined that the number of antibody molecules required for neutralization were approximately 14, 30, 72, and 36 molecules for the four mAbs 5B6, 3, 6, and 9 studied, respectively (Roden *et al.,* 1994). An average of about 38 bound IgG molecules is therefore required to neutralize a BPV virion. Complexes of mAb 5B6 and 9 with the major capsid BPV protein L1 have been analyzed by cryo-EM. It was shown that mAb 5B6, which apparently requires a slightly lower occupancy for neutralization, binds only to hexavalent capsomers, whereas mAb 9 also binds to pentameric capsomers (Booy *et al.,* 1998). MAb 9 binds monovalently at the tip of the capsomers and is highly contorted, projecting the Fc domain at a 120° angle into the space between the capsomers, while the free Fab domain projects outward. MAb 9 provides a dense extended antibody coat, which is consistent with its ability to inhibit attachment of BPV to the target cell. mAb 5B6 binds deeply between the hexavalent capsomers and adopts a more

linear conformation. MAb 5B6 does not inhibit virion attachment efficiently and likely interferes with a postattachment step (Booy *et al.,* 1998). It is possible that mAb 5B6 may coat the virus more extensively by its lower molecular profile and may therefore be more effective at a somewhat lower level of occupancy.

The relatively low number of antibodies required to neutralize some viruses compared to others has generated much discussion. Critical site hypotheses have been widely used to explain neutralization. However, how critical "neutralization-relevant" spikes differ from other "neutralization-irrelevant" spikes has not been defined, and although molecular rearrangements in the capsid on nonenveloped viruses may provide a possible mechanism, it is unclear how signals would be transmitted between loosely attached spikes in the membrane of an enveloped virus. Some studies have suggested that binding of a neutralizing antibody, for example, to the hemagglutinin (HA) of influenza virus, transmits a signal across the envelope, effecting neutralization (Possee *et al.,* 1982; Taylor *et al.,* 1987). A molecular mechanism for this is unknown.

A much simpler hypothesis favored by us is that neutralizing antibodies generally act by coating the virus surface, thereby interfering with critical steps in the infectious pathway, such as obstructing interactions with cell surface receptors or interfering with molecular rearrangements required for virus–cell fusion. Figure 2 provides a schematic representation of the size of the viruses discussed above relative to antibody. The average number of antibody molecules required to neutralize the viruses depicted by increasing size from left to right is 4, 5–6, 38, 70, and 225. A plot of the number of antibodies required for neutralization as a function of the average surface area for each of the viruses is shown in Fig. 3a. The straight line demonstrates that a roughly equivalent fraction of the virion surface, independent of the size of the virion, must be bound by antibody for neutralization to occur.

To calculate the average surface area on a virion associated with each antibody molecule at neutralization, we determined the slope of the line in Fig. 3. The line in Fig. 3a can be described by the formula: $N = 0.0033 \times A$, in which N is the number of IgG molecules required for neutralization, and A is the total virion surface area. It follows that each antibody molecule is associated with an average of 300 nm^2 virion surface area at neutralization. It is not unreasonable to assume that an antibody molecule can provide steric hindrance for an area this size. A 300-nm^2 area is covered by a circle with a radius of about 9.7 nm. The length of an antibody molecule is about 12.5 nm, of which approximately 7 nm is provided by the Fc tail which has a high degree of translational and rotational flexibility relative to the Fab domains. This indicates that it is not unlikely that an antibody, even when bound bivalently, whereby movement of the Fab domains is restricted, could hinder the interaction of virion surface proteins with critical sites on the target cell in an area surrounding the binding site or, alternatively,

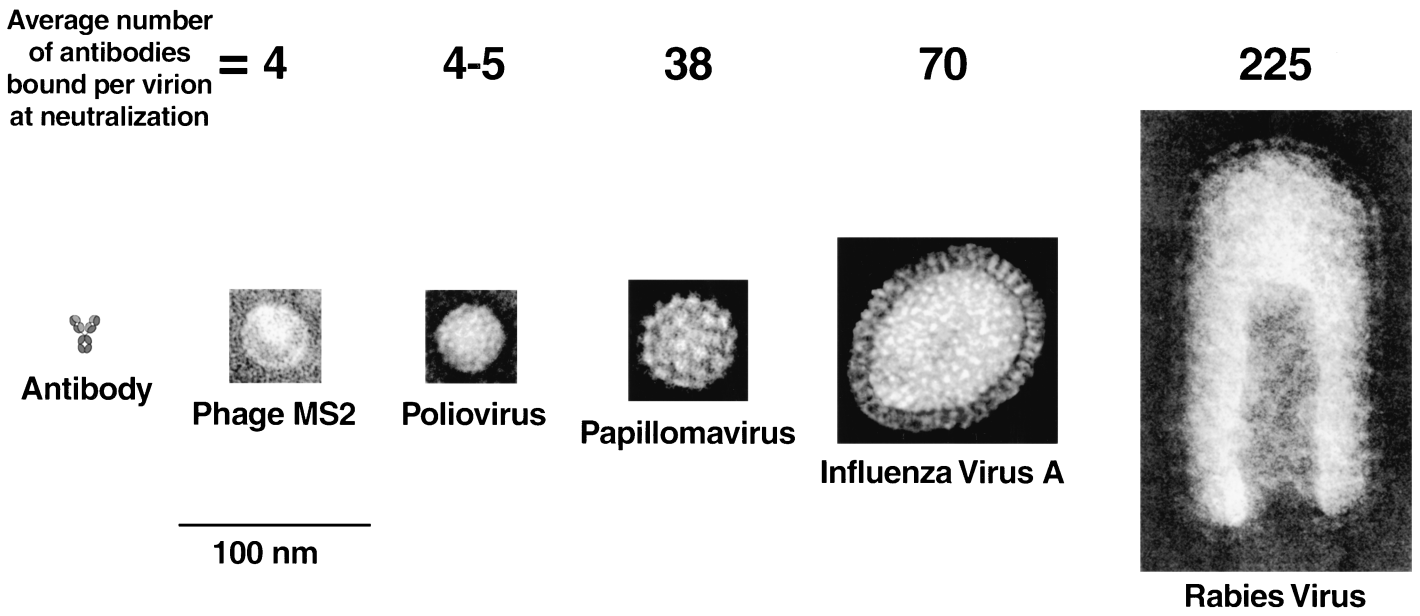

FIG. 2. Schematic representation of virion size relative to antibody. Electron microscopic images of each of the viruses considered are depicted on scale relative to the size of an antibody molecule. Viruses with a larger average virion size appear to require the attachment of more antibody molecules to be neutralized. Clearly, however, viruses are generally neutralized well before all available mAb binding sites have been occupied. Saturation of binding sites, for example, only occurred with 30 IgG molecules bound per poliovirus and 1080 IgG molecules bound per rabies virus particle (Flamand *et al.*, 1993; Icenogle *et al.*, 1983). Electron microscopic images were provided by Dr. Linda M. Stannard, University of Cape Town, South Africa (poliovirus, influenza virus and polyoma virus); Dr. H. W. Ackermann, Université Laval, Quebec, Canada (bacteriophage MS2); Dr. S. K. Vernon, Wyeth Laboratories, Philadelphia, PA (rabies virus). Rabies virus reprinted from Vernon *et al.* (1972) with permission granted by Academic Press, Orlando, FL.

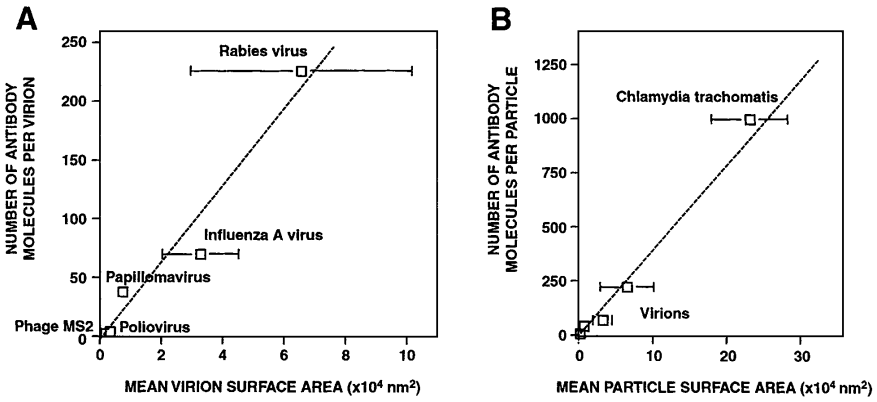

FIG. 3. Antibody occupancy of virion surface binding sites at neutralization. To study the observation in Fig. 2 in more detail, we plotted the number of antibodies required for neutralization as a function of the average surface area for each of the viruses shown. A range of virion surface areas is indicated as preparations, particularly of enveloped viruses, are typically heterogeneous with respect to size and shape. (a) The straight line demonstrates a linear relationship. (b) This relationship can be extrapolated to the large pathogen, the intracellular bacterium *Chlamydia trachomatis*.

hinder the formation of intermediates in the viral entry process such as fusion complexes.

We believe that the available data, in agreement with the occupancy model, indicate that antibody-covered virions are either neutralized or not, and that there is no convincing evidence for any intermediate states. Virions covered with antibodies at low occupancy may infect their target cells more slowly, as discussed above. Such particles, however, are still infectious and therefore do not represent a neutralization intermediate, in our view. Once antibody coating proceeds beyond a critical density, which appears to equal a relatively constant number of antibody molecules per unit of virion surface area, the virion is rendered noninfectious. Neutralization is expected to be reversible by dissociation of the antibody–virion complexes in most cases. Neutralization of a virus population can thus be understood in similar terms. The partial neutralization of a homogeneous virus stock at a certain concentration of antibody (e.g., 50%) simply represents a Poisson distribution of antibody molecules attached to virions at a given point in time. The fraction of the viruses coated with antibody molecules below the neutralization threshold is fully infectious, whereas the complementary fraction is fully neutralized. Because antibody typically is in vast molar excess over virus in a neutralization reaction, the average number of binding sites occupied at a given antibody concentration, and thus the fraction of neutralized virus, is predicted to be primarily determined by antibody affinity for the virion.

The antibody occupancy at neutralization for the intracellular bacterium *Chlamydia trachomatis* has been determined in a study which is much less quantitative than the studies on viruses discussed above. We include it in our discussion here, as it is the only study of which we are aware to provide an estimate of the number of antibody molecules required to neutralize a nonviral pathogen. The study found that approximately 1000 antibody molecules of the strongly neutralizing mAb UM-4 were required to reduce infectivity of infectious elementary bodies by 50% (Peeling and Brunham, 1991). (A higher number was given for a weakly neutralizing antibody, but we consider this less reliable, since the study assumed that all the antibody in an equilibrium mixture was bound to bacteria.) Interestingly, the *C. trachomatis* data can be fit by extrapolation of the line described for viruses in Fig 3a (Fig 3b).

The occupancy model suggests that one mechanism by which virus could seek to escape neutralization by antibody would be to decrease the number of neutralizing epitopes on the viral surface. Such a mechanism has indeed been described for human cytomegalovirus (HCMV) (Li *et al.,* 1995). A neutralization escape mutant was isolated from a patient who was being treated with a neutralizing human antibody against the HCMV surface glycoprotein gH. The escape mutant was unstable when cultured in the absence of anti-gH neutralizing antibody and displayed a resistant phenotype against a number of distinct neutralizing antibodies and a polyclonal serum against gH. Biochemical analysis revealed that the escape mutant displayed a marked decrease in the amount of gH expressed on the surface. Interestingly, this mechanism of resistance would be consistent with the large differences in susceptibility to neutralization of clinical HCMV isolates, and might be a mechanism by which the virus persists in the presence of neutralizing antibody in the serum (Li *et al.,* 1995). If antibody occupancy indeed plays a major role in neutralization as we suggest, then down regulation of surface-specific molecules on certain viruses may be a more common mechanism of neutralization resistance than currently thought. However, as in the case of HCMV, it may be necessary to culture fresh clinical isolates in the presence of neutralizing antibody to observe these effects, since passaging would allow rapid reversion to wild type.

The occupancy model also suggests that Fab fragments may neutralize a viral particle less efficiently than the corresponding whole antibody, as steric hindrance by the Fab is provided at a significantly reduced level. Generally, a ~1- to 50-fold difference is found in neutralization potency between whole IgG and Fab, which has been explained by differences in avidity (reviewed in Parren and Burton, 1997). Some examples from the literature, however, indicate that a loss of steric hindrance may also play a role in this decrease. Icenogle *et al*. (1983) showed that treatment of neutralized poliovirus particles at low occupancy (an occupancy of the order of 6 antibody molecules per poliovirus particle reducing infectivity of the virus stock to 28%) with papain to remove the IgG Fc tail reactivated the infectivity. It was shown that the Fab fragments were still bound to the

virion, however, as addition of an anti-antibody again reduced infectivity to the initial neutralized level (Icenogle *et al.,* 1983). Similar observations were made by Emini *et al.* (1983b). For the paramyxovirus SV5, Merz *et al*. (1981) have shown that anti-HN antibody completely neutralized the virus, whereas Fab fragments could only cause partial neutralization, and a relatively large fraction of nonneutralizable virus remained. The neutralization mechanism of this antibody did not appear to involve aggregration or cross-linking, excluding this as the basis for the difference.

In agreement with the model proposed here, it has been shown that the neutralization potency of mAbs against the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) correlated with occupancy as potent neutralizing mAbs stained virions in immunoelectron microscopy more strongly than weakly neutralizing mAbs (Weiland *et al.,* 1999).

The occupancy model may finally explain the reduction in neutralization rate as a function of time in kinetic experiments. As viruses are more densely decorated with antibody molecules, the reaction rate will start to decrease, as incoming antibody will be sterically hindered by antibody already bound to the virus. Such steric hindrance has been shown to exist by kinetic neutralization experiments on herpes simplex virus (HSV) performed by Ashe and Notkins. These authors demonstrated a drop in the neutralization rate constant as neutralization progressed, the magnitude of which correlated with the level of sensitization (Ashe and Notkins, 1967). Kinetic neutralization studies using HSV presensitized with a neutralizing antibody, furthermore, showed that neutralization rate constants were strongly reduced if the subsequent neutralization reaction was performed with the whole antibody, but were only mildly reduced when performed with the Fab fragment (Ashe *et al.,* 1969). Therefore, under the conditions used, the antibody already bound to the virion reduced the binding rate of whole antibody relative to that of the smaller Fab fragment, presumably by a steric hindrance mechanism.

K. A Molecular Model for Picornavirus Neutralization

It has been shown that the attachment of between 4 and 7 IgG molecules can lead to the neutralization of a picornavirus particle. In particular, the binding of about 6–7 IgG molecules to HRV prevents its attachment to the target cell thereby neutralizing the virus. (Colonno *et al.,* 1989; Icenogle *et al.,* 1983; Smith *et al.,* 1993).

To visualize how the binding of a small number of IgG molecules to the surface of a 30-nm picornavirus particle can impact the virus–receptor interaction at the cell surface, we prepared a molecular model of a picornavirus with a low number of antibodies bound. Poliovirus capsid was modeled from coordinates in the Protein Data Bank (PDB)(Berman *et al.,* 2000) entry 1dgi (He *et al.,* 2000). Antibody (human IgG1) was modeled from PDB entry 1igt (Harris *et al.,* 1997) and coordinates from Erica Ollmann Saphire (personal communication and

Ollmann Saphire *et al.,* 2001). Molecular surfaces were calculated with the program MSMS (Sanner *et al.,* 1996) using C alpha atoms of 4 Å radius and a reduced viral surface of 1.4 million triangles. Viral surface covered by antibody was calculated with python (Sanner, 1999) using a cylinder of 90 Å radius penetrating the viral capsid. Surfaces and models were visualized in AVS and PMV (Sanner *et al.,* 1999), while artistic impressions of IgG domain movements were rendered in Adobe Photoshop.

In the diagram in Fig 4, three IgG molecules are shown interacting with the visible half of the surface area of a picornavirus particle. This level of coating is close to that predicted to neutralize the virus. The considerable rotational flexibility of the Fc region (with dimensions of $7 \times 5.7 \times 2.4$ nm) is demonstrated for one IgG molecule (Fig. 4a). Taking the flexibility of Fc and the translational flexibility of the antigen-bound Fab fragment into account, the antibody could be thought to hinder the attachment of virus to cellular receptors in a significant area surrounding the antigen-binding site, as shown in Fig 4b. As the initiation of productive infection requires the interaction of virus with a number of receptor molecules in a localized area (see also Fig. 6), it can be envisioned that the binding of a relatively small number of antibody molecules to a picornavirus particle can effectively interrupt attachment and initiation of infection via steric hindrance and geometric constraints.

L. The Case of Influenza Virus

Antibodies against influenza virus neuraminidase (NA) and matrix protein 2 (M2) have been used as an example of binding but nonneutralizing antibodies. The surface of influenza viruses contains three types of spike proteins, namely HA, NA, and M2, inserted in a host cell-derived membrane (Murphy and Webster, 1996). Whereas antibodies against HA neutralize influenza, antibodies against NA and M2 do not (Kilbourne *et al.,* 1968; Webster and Laver, 1967; Zebedee and Lamb, 1988). In the presence of antibodies against NA and M2, however, virus growth *in vitro* and *in vivo* is reduced, indicating that these antibodies do recognize these proteins on the surface of the virus or infected cells. The activities observed are most likely the result of reduced virus release and antibody-mediated destruction of infected cells (Murphy *et al.,* 1972; Webster and Laver, 1967; Zebedee and Lamb, 1988).

The absence of neutralizing activity of antibodies against NA and M2 can be explained by their abundance and distribution on the viral surface, and is in direct agreement with the occupancy hypothesis. The NA and M2 proteins are far less abundant on the viral surface than the HA protein; generally there are four to five times more HA than NA spikes, and NA spikes furthermore seem to be organized in patches. Each virus usually contains only a few copies of the M2 protein (Lamb and Krug, 1996; Murphy and Webster, 1996). Therefore, although antibodies against NA and M2 may bind to these proteins on the viral

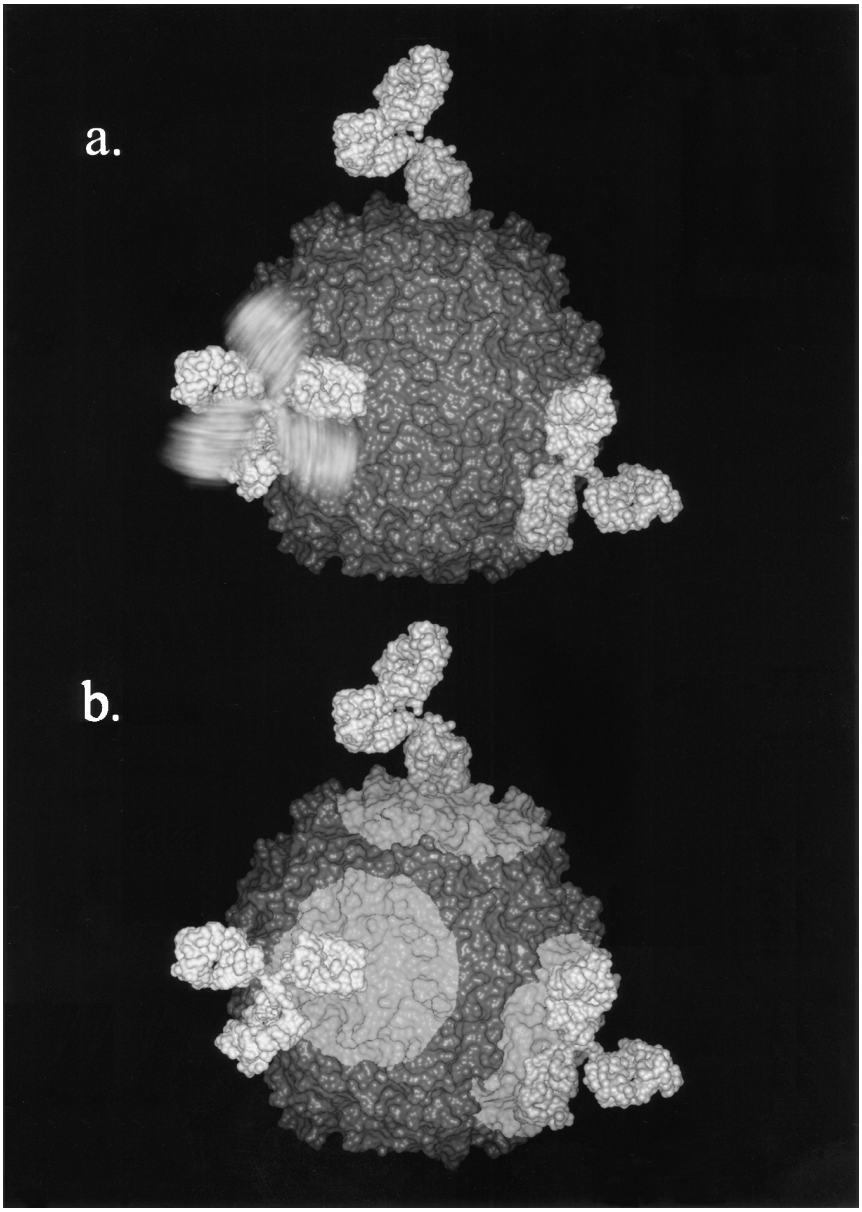

FIG. 4. Picornavirus neutralization. (a) Picornavirus particle with three IgG molecules (gold) attached to approximately half of the virion surface area. Rotational flexibility of the Fc region is demonstrated for one IgG molecule. (b) Region of virus particle surface (green) subject to direct obstruction by a bound IgG molecule. Binding of an average of about 5–6 IgG molecules/picornavirus particle results in neutralization. This number of bound antibody molecules prevents attachment of HRV to target cells. Surfaces were calculated as described in Sanner *et al.* (1996). This diagram was kindly prepared by Erica Ollmann Saphire.

surface, the maximum level of occupancy achievable is relatively low and likely to remain below the level required to inhibit infectivity. This is reminiscent of the observations with the minor acid-inducible form of the rabies spike protein described above (Raux *et al.,* 1995).

M. Neutralization by Antibodies Against Host Cell-Derived Proteins Incorporated into Virions

Many instances of viruses, particularly retroviruses, that incorporate nonviral host cell-derived proteins into their viral envelopes have been described. HIV-1 has been shown to incorporate HLA class I and class II molecules, ICAM-1, LFA-1, and CD44 (Arthur *et al.,* 1992, 1995; Capobianchi *et al.,* 1994; Gelderblom *et al.,* 1987; Hoxie *et al.,* 1987; Orentas and Hildreth, 1993; Saarlos *et al.,* 1997). Simian immunodeficiency virus (SIV) and FIV, furthermore, may also incorporate HLA antigens (Arthur *et al.,* 1992; Lee *et al.,* 1982). This may have important consequences, as macaques immunized with human cells or HLA-DR, for example, can be protected from challenge with SIV grown in human host cells (Arthur *et al.,* 1995; Stott, 1994). It has been suggested that cellular adhesion molecules associated with HIV-1 may have a function in increasing adherence to the target cell and thus enhance virus entry; a particularly well-studied example of which is ICAM-1 (Rizzuto and Sodroski, 1997). Antibodies against such host cell-derived molecules can have neutralizing activity; for example, ICAM-1-expressing HIV-1 virions can be completely neutralized by an antibody against ICAM-1. Interestingly, the neutralizing antibody activity observed is comparable in magnitude to that of "classic" neutralization. The neutralizing effect, therefore, is far greater than can be explained by the loss of adhesion provided by virion ICAM-1 in contact with its cognate receptor LFA-1 on the target cell (Rizzuto and Sodroski, 1997).

Antibodies against HLA class I and II have been shown to neutralize SIV, in which neutralization susceptibility appears to be determined by the number of HLA molecules present on the virion (Vzorov and Compans, 2000). Incorporation of influenza virus HA molecules on phenotypically mixed SIV virions, furthermore, renders the virions susceptible to neutralization by antibodies against influenza HA (Vzorov and Compans, 2000). This was also observed for phenotypically mixed virions of VSV with FPV, Sendai, and measles virus (Kimura, 1973; Wild *et al.,* 1975–1976; Zavada and Rosenbergova, 1972), as discussed above.

Neutralization of viruses by antibodies to foreign proteins on the viral surface supports the occupancy model. Clearly, viruses can be neutralized by the binding of antibodies to sites on the viral surface which do not directly serve a function in the infectious process (e.g., influenza HA on SIV). Coating of the viral surface with antibodies against such molecules, and subsequent steric interference with the virion–host cell interaction required to initiate infection, is an attractive hypothesis to explain these observations.

N. Inhibition of Infection by Anti-Receptor Antibodies

If the interruption of viral infection is primarily mediated by an occupancy and steric hindrance mechanism as we propose, then binding of antibodies to the viral receptor on the target cell should also inhibit the initiation of infection. We would predict that the ability of antireceptor antibodies to inhibit infection would then also be a function of occupancy and mostly independent of the epitope recognized. One of the best studied examples is probably the inhibition of HIV-1 infection by antibodies against its primary receptor CD4. CD4 is a molecule of four Ig-like domains. The first two domains project from the cell surface and are connected by a hinge to the last two domains that have been suggested to lie parallel to the cellular membrane (Wu *et al.,* 1997). The gp120-binding site has been mapped to the first, most membrane-distal domain of CD4 (Arthos *et al.,* 1989).

A large array of mAbs against distinct epitopes on CD4 have been studied for their ability to inhibit gp120 binding to $CD4^+$ cells and HIV-1 infection, and most antibodies in fact were found to inhibit infection (Healey *et al.,* 1990; Sattentau *et al.,* 1986). It is interesting that even antibodies mapped to the third domain, which do not affect the interaction between CD4 and gp120, effectively inhibit infection. A comparison of mAbs against the first and third domains indicated that these antibodies inhibited HIV-1-induced syncytium formation for a range of isolates with similar potency. Inhibition of HIV-1 infectivity required that binding saturation of all available binding sites on the target cell surface was achieved. This inhibition was not correlated with a down regulation of CD4 during the experiment (Healey *et al.,* 1990). The potency of mAbs to the third domain of CD4 is noteworthy, given that this domain is not required for HIV-1 infection, as shown by replacement of the last two domains of CD4 with those of CD8, with retention of receptor activity (Bedinger *et al.,* 1988). One antibody, which probably binds to domain 3 (OKT4), did not inhibit HIV-1 infection. However, this may reflect the relatively poor affinity of this antibody for CD4 (Healey *et al.,* 1990; Truneh *et al.,* 1991). One antibody against the fourth domain of CD4 does not appear to block HIV-1 infection (Healey *et al.,* 1990; Truneh *et al.,* 1991). Even though antibodies against the third domain of CD4 inhibit HIV-1 infection, they do not appear to inhibit virion binding to $CD4^+$ T cells (Healey *et al.,* 1990; Ugolini *et al.,* 1997). Interestingly, it has been shown that most anti-CD4 antibodies, irrespective of the epitope recognized, are able to interrupt major histocompatibility complex (MHC) class II-dependent T cell activation. Similar to their HIV-1 inhibitory activity, significant inhibition only occurs at near-saturating antibody concentrations (Merkenschlager *et al.,* 1990).

The studies described above appear to show that inhibition of HIV-1 infection by anti-CD4 antibodies is dependent on the saturation of all available binding sites and is mostly independent of epitope recognized. However, a difference

exists between antibodies against the virus and CD4 in their ability to inhibit virus–cell binding. Whereas all neutralizing antibodies against gp120 inhibit virus attachment, only antibodies which bind in the direct vicinity of the high-affinity gp120 binding site on CD4, and not antibodies to more-distant epitopes, including those in the first domain, inhibit attachment (Truneh *et al.,* 1991; Ugolini *et al.,* 1997). It may be that antibody affinity plays a role in the observed difference, as the inhibitory CD4 mAbs tested were of higher affinity than the noninhibitory CD4 mAbs (Healey *et al.,* 1990; Truneh *et al.,* 1991). Alternatively, it may be a consequence of differences in antibody coating densities. The spike density on the virus, and therefore its antibody coating density, is several orders of magnitude greater than the receptor and resulting antibody coating density of the target cell. It is therefore conceivable that a virus densely coated with a dense antibody cannot approach the target cell sufficiently to initiate binding, whereas viruses are not inhibited in their approach to separated clusters of CD4 molecules with antibodies attached to epitopes distant from the gp120 binding site. Presumably, however, the antibodies attached to CD4 then perturb the necessary spatial organization of the multiple envelope–receptor contacts and conformational changes required to initiate a productive infection event. Notably, it has been suggested that these same antibodies can interrupt the precise spatial organization of CD4-T cell receptor and accessory molecules required for triggering T cell proliferation (Merkenschlager *et al.,* 1990).

Following CD4 binding, HIV-1 requires the interaction with a chemokine receptor, belonging to the seven-transmembrane G-coupled receptor family, as an obligatory coreceptor for HIV-1 entry into the target cell. While a large number of chemokine receptors can serve as coreceptors for HIV-1 entry, the majority of isolates use either CXCR4 or CCR5 as their major coreceptor (see Berger, 1997, for a review). A recent study investigated the inhibition of HIV-1 infection by antibodies against a number of epitopes on CCR5 (Lee *et al.,* 2000). An antibody against the second extracellular loop (ECL2-A epitope), mAb 2D7, effectively inhibited HIV-1 infection and gp120 and chemokine binding to receptor. All other antibodies studied and directed against a different epitope on ECL2 (ECL2-B epitope), the N-terminus and epitopes involving multiple loops, blocked infection more poorly or not at all, and varied in their ability to inhibit gp120 and chemokine binding to receptor. MAbs against the N-terminus blocked gp120 binding better than chemokine binding, while the reverse was found for antibodies against the ECL2-B epitope. The affinities of these antibodies for CCR5 varied but were equal or greater than that of mAb 2D7 in some cases (Lee *et al.,* 2000). Interestingly, it was found that CCR5 may exist in multiple conformational forms on the cell surface. MAb 2D7, the antibody with the strongest neutralizing activity, recognizes an epitope which is expressed on a much greater proportion of the CCR5 molecules than all the other mAbs tested, which appear to bind to only a fraction of the CCR5 molecules expressed

(Lee *et al.,* 2000). Therefore, a correlation between inhibition of HIV-1 infection and occupancy of binding sites exists, similar to the studies discussed above. We suggest that most antibodies against CCR5 are not effective in inhibition of HIV-1 infection, as they only recognize a subset of CCR5 molecules on the cell surface and therefore cannot saturate potential HIV-1 coreceptor binding molecules.

III. Complement-Mediated Virolysis

The binding of antibodies to the viral surface may lead to the activation of complement, which is dependent on the isotype of the antibody and the spacing of the epitope recognized. Complement activation may lead to virus inactivation at subneutralizing concentrations. A number of enveloped viruses including HIV-1, HCMV, and vaccinia virus (Smith, 1999; Spiller *et al.,* 1997) carry complement regulatory molecules on their surfaces. These are acquired from the host cell membrane during budding (e.g., CD46, CD55, and CD59), or recruited from plasma by attachment envelope glycoproteins, as in the case of factor H and HIV-1 (Saifuddin *et al.,* 1994, 1995; Stoiber *et al.,* 1997; Sullivan *et al.,* 1996). These regulatory molecules promote the resistance of virions to virolysis by inhibiting full activation of the complement cascade and thereby provide the virus with a means to escape inactivation by the binding of relatively low amounts of antibody. Primary isolates of HIV-1 have been found to be more resistant than TCLA viruses to complement-mediated virolysis (Saifuddin *et al.,* 1995; Spear *et al.,* 1990; Sullivan *et al.,* 1996). The resistance of primary isolates to virolysis was found to directly correlate with the poor exposure of antibody binding sites on the primary virus envelope (Takefman *et al.,* 1998). A difference has been noted between HIV-1 primary isolates (grown in PBMC *in vitro*) and plasma virus in their sensitivities to complement-mediated neutralization (Sullivan *et al.,* 1996, 1998a; Takefman *et al.,* 1998). Plasma virus is considerably more sensitive to antibody-dependent, complement-mediated virolysis (Sullivan *et al.,* 1996; Takefman *et al.,* 1998). An explanation can be found in low expression levels of complement regulatory factors, in particular CD46 and CD55, on the plasma virion surface, compared to on a primary isolate virion (Sullivan *et al.,* 1996). Diminished expression of these complement regulatory molecules on plasma virions may be explained by their decreased expression on $CD4^+$ lymphocytes from HIV-1 infected individuals, since the viral membrane is derived from the cell membrane (Lederman *et al.,* 1989; Weiss *et al.,* 1992).

Some viruses, such as orthopoxvirus and herpesvirus, express viral complement regulatory molecules either on their surface or in soluble form that may contribute to viral evasion (e.g., Fodor *et al.,* 1995; Kotwal *et al.,* 1990; Rother *et al.,* 1994; Tortorella *et al.,* 2000). Orthopoxviruses use both these strategies. First, the EEV, which is responsible for cell-to-cell and long-range spread of

orthopoxvirus infection (Blasco and Moss, 1992), expresses a viral protein on its membrane, B5R, which has homology to complement control proteins and may provide protection against complement-mediated virolysis of EEV (Engelstad *et al.,* 1992; Isaacs *et al.,* 1992; Vanderplasschen *et al.,* 1998). Vaccinia virus, cowpox, and variola virus secrete complement control proteins that inhibit complement activation via inhibition of C3 convertase activity (Kotwal *et al.,* 1990; Rosengard *et al.,* 1999; Tortorella *et al.,* 2000).

IV. Antibody-Mediated Phagocytosis

Virions coated by antibody may be eliminated by phagocytosis followed by inactivation in an intracellular compartment within the phagocyte. This process has been described for the picornavirus foot-and-mouth disease virus (FMDV). FMDV sensitized with antibody at subneutralizing concentration was actively phagocytosed *in vitro* by monocytes and macrophages (McCullough *et al.,* 1988). Phagocytosis *in vivo,* furthermore, appears to play a role in protection against FDMV infection (see below).

V. Antibody-Mediated Cytotoxicity

Antibodies which bind effectively to viral proteins expressed on the surface of infected cells may trigger complement-mediated cytotoxicity (CMC) and ADCC and thereby provide protection against viral infection *in vivo*. The antibody isotype and spacing of the epitopes are important parameters for the efficient induction of effector functions by antibody. The dominant human IgG isotype IgG1 binds well to IgG Fc receptors and is capable of activating complement via the classical pathway (Burton and Woof, 1992; Parren, 1992). CMC and ADCC can be mediated by neutralizing antibodies (directed against viral proteins also expressed on the virion) as well as nonneutralizing antibodies (directed against viral proteins exclusively expressed on infected cells). The role of CMC and ADCC in protection against viral infection will be discussed in specific examples below. Generally, however, it may be expected that neutralizing antibodies provide stronger protection than nonneutralizing antibodies, as the former can act against free virions as well as against infected cells (see below).

A number of viruses, such as HCMV, HSV-1, HSV-2, and varicella-zoster virus (VZV) express virally-encoded Fc receptors on their surface. It has been suggested that viruses decorated with antibody use these receptors to bind Fc, thereby obscuring this part of the immunoglobulin (Ig) molecule from cellular Fc receptors or complement (Lubinski *et al.,* 1998; Tortorella *et al.,* 2000). This indeed would seem an effective mechanism by which virions with neutralizing antibody bound at low occupancy may escape inactivation *in vivo*.

VI. Intracellular Neutralization

Polymeric IgA and IgM involved in mucosal immunity may make use of a specialized route to neutralize viruses or prevent viral entry into the host. These antibodies are actively transported over the mucosal epithelium after binding to the polymeric Ig receptor and may, during transport, contact and neutralize transcytosing viruses. Sendai virus added to the apical side of an epithelial cell monolayer was shown to interact intracellularly with polymeric IgA added basolaterally, and viral titers were reduced in cellular supernatant and lysate (Fujioka *et al.,* 1998; Manzanec *et al.,* 1992). Manzanec *et al*. demonstrated neutralization of influenza virus via a similar mechanism (Manzanec *et al.,* 1995; reviewed in Kato *et al.,* 2000). Studies by Bomsel *et al*. (1998) have shown that that transcytosis of primary HIV-1 isolates over tight epithelium can be blocked by dimeric immune IgA and IgM. It was shown that basolaterally internalized antibodies met transcytosing virions at an intracellular compartment, thereby redirecting the virions back to the mucosal compartment (Bomsel *et al.,* 1998). Although the affinity for virions of the polyclonal dimeric IgA and IgM, purified from serum from infected individuals, is likely to be low, efficient binding may be occurring by a multivalent interaction at relatively high concentrations within the intracellular compartment. Polymeric IgA and IgM therefore may bind to virus in an intracellular compartment, neutralizing infectivity or redirecting transcytosis.

VII. Mechanisms of Antibody Protection *in Vivo*

A. Introduction

A number of the mechanisms described for antiviral activity *in vitro* may operate *in vivo*. These are summarized in Fig. 5. They include mechanisms that act upon free virions, such as neutralization, and those that act upon infected cells, such as CMC and ADCC. As discussed above, in our view, neutralization is mediated by antibodies that bind to envelope molecules on the virion surface and effectively coat the virion, preventing viral attachment and/or fusion. Such antibodies can also bind to envelope molecules expressed on the surface of infected cells and thereby trigger elimination of these cells by Fc-mediated effector systems. Nonneutralizing antibodies can similarly bind to viral proteins expressed on infected cells to produce antiviral activity. These proteins can include envelope molecules in forms that are not expressed or only expressed in relatively low amounts on virions. Finally, there is evidence of special instances where binding of antibodies to infected cells appears to suppress viral replication without leading to cell destruction.

For many viruses, there is a correlation between levels of serum antibody neutralizing activity *in vitro* and *in vivo* protection. Are these antibody levels responsible for the protection or merely correlated with it? The classical approach

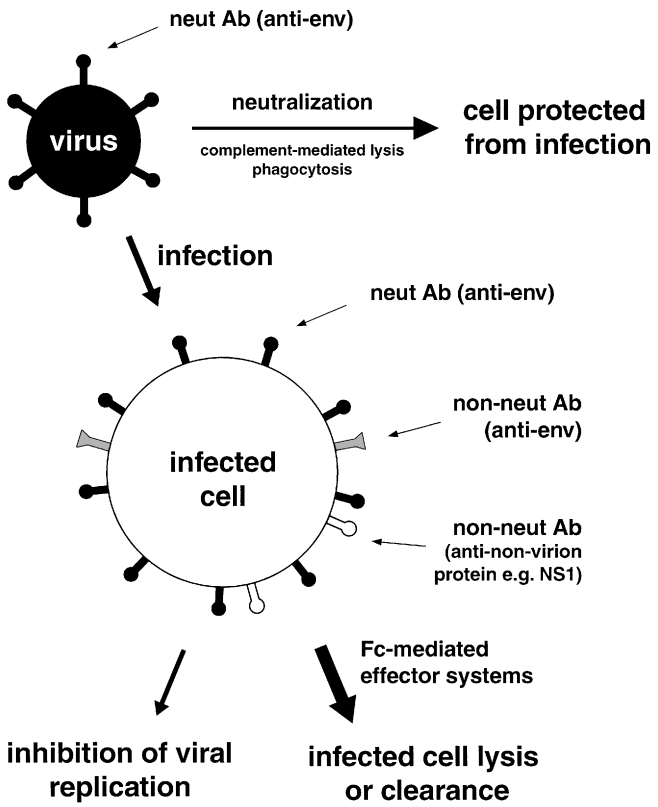

FIG. 5. Mechanisms of antiviral antibody activity *in vivo*. Antiviral activity of antibodies can be separated into activity against virions and activity against infected cells. Neutralization is mediated by antibodies that bind well to molecules on the virion surface and effectively coat the virion, thereby preventing viral entry. Antibody-mediated effector mechanisms such as complement-mediated lysis and phagocytosis can aid in virus inactivation. Antibodies against envelope spikes of an enveloped virus may also bind to envelope molecules on the surface of infected cells and thereby trigger elimination of these cells by Fc-mediated effector mechanisms. Nonneutralizing antibodies can similarly bind to viral proteins on infected cells to produce antiviral activity. These proteins can include molecules in forms that are not expressed or only expressed in low amounts on virions.

to this question has been to passively transfer serum antibodies from immune animals to naïve animals and determine the protection provided. In many cases (Chanock *et al.,* 1993), passive transfer does provide protection from disease. There is also evidence in humans of the ability of passively transferred antibodies to protect against disease arising from a number of viral pathogens including RSV, hepatitis A virus, measles virus, poliovirus, VZV, and variola virus (Chanock *et al.*, 1993). In most cases, it has not been determined whether antibodies provide

sterile protection. It may be that, in many cases, antibodies limit infection, and cellular and innate immunity are also required in protection from disease.

Antiviral monoclonal antibodies permit a more quantitative investigation of the relationship between neutralization *in vitro* and protection *in vivo*. Clearly, the comparison presents difficulties in that, even if very similar antiviral mechanisms operate *in vitro* and *in vivo,* the latter involves a variety of factors for which it is difficult to control, and which may not be easily reproduced *in vitro*. For instance, the host cell type used *in vitro* may differ from the activation/maturation state of the cell type infected *in vivo*. In fact, cell lines are often used *in vitro*. Other factors and molecules may be influential *in vivo* but absent *in vitro*. Viruses with distinct characteristics may be selected *in vivo* and *in vitro,* leading to aberrant conclusions; HIV-1 and lactate dehydrogenase-elevating virus (LDV) are striking examples, which will be discussed below. Thus, caution in any comparison is appropriate, but nevertheless it is important to ask how well neutralization of mAbs *in vitro* correlates with protective activity *in vivo*. It should be pointed out that a good correlation does not necessarily prove that the *mechanisms* of *in vitro* neutralization and *in vivo* protection are the same. Since neutralization is so strongly correlated with virion binding, any mechanism that was strongly correlated with such binding (e.g., complement-mediated virolysis) could in theory be responsible. However, in many cases, *in vivo* activity has been shown to be independent of the Fc part of the IgG molecule, implying that the neutralization blocking mechanism is probably paramount.

B. Retroviruses

For HIV-1, we have reported (Gauduin *et al.,* 1997; Parren *et al.,* 1995, 1997a) that sterile protection of hu-PBL-SCID mice from virus challenge with laboratory-adapted or primary viruses requires serum mAb concentrations one to two orders of magnitude greater than those required for 90% *in vitro* neutralization, that is, concentrations corresponding to essentially complete virus neutralization *in vitro*. TCLA viruses, which are much easier to neutralize *in vitro,* are correspondingly much easier to protect against *in vivo*. Similar findings with regard to the relationship between neutralization and protection are apparent for challenge of macaques with chimeric SIV/HIV (SHIV) viruses (Baba *et al.,* 2000; Mascola *et al.,* 2000; Shibata *et al.,* 1999). In one instance of mucosal challenge, partial protection was described for a serum mAb concentration corresponding to only about 90% neutralization *in vitro* (Mascola *et al.,* 1999). Recently, however, we have found that protection from mucosal challenge with an R5 SHIV virus required mAb concentrations capable of neutralizing essentially all of the challenge virus (Parren *et al.,* 2001b). In fact, the results in the macaque model closely paralleled those in the SCID mouse study: Since effector systems are likely to be considerably more efficient in the former than the latter model, the results imply that effector systems are not essential in protection against HIV-1.

Research on the biology of tumors induced by murine leukemia viruses (MuLV) has shown that antibodies against MuLV antigens expressed on the surface of malignant-infected cells can provide protection against tumor growth. Antibodies against Gross virus cell surface antigens provided complete protection against challenge with syngeneic Gross cell surface antigen-positive leukemia cells. Lysis of these cells by CMC was demonstrated *in vitro* (Old *et al.,* 1967). The Gross virus cell surface antigen was later found to be identical to MuLV glyco-gag, which is a glycosylated version of the gag polyprotein that is expressed on the cell surface but is not incorporated into virions (Ledbetter and Nowinski, 1977). It should be noted that the antisera used by Old *et al*. (1967) also contained neutralizing antibodies against Gross virus, so that it is uncertain that all of the protective effect was mediated by antibodies against glyco-gag. Similarly, however, nonneutralizing mAbs against Moloney MuLV (M-MuLV), which react with a Friend–Moloney–Rauscher virus group-specific antigen expressed on the surface of infected cells but not on virions, protect against tumor growth (Lamon *et al.,* 1987).

Pincus *et al.* (1995) more recently showed that nonneutralizing antibodies against MuLV (glyco-)gag delayed onset of MuLV pathogenesis. An antibody against gag also delayed disease onset as a Fab fragment (albeit less effectively), indicating that clearance of infected cells by Fc-mediated effector functions cannot wholly explain the protective effect. It has been shown that MuLV glyco-gag plays an important role in viral spread and pathogenesis (Corbin *et al.,* 1994; Portis *et al.,* 1994). Occupancy of glyco-gag on the cell surface by antibody in the form of whole IgG as well as Fab fragment may therefore be able to interfere with glyco-gag function, thereby providing protection by limiting virus spread without directly clearing infected cells.

Protection against MuLV challenge as expected is also provided by antibodies against envelope which are more effective than antibodies against gag and show a good correlation between neutralization titer and the degree of protection provided (Pincus *et al.,* 1995). The virus used in these studies was a recombinant Friend MuLV (F-MuLV) with the *env* gene of the neurovirulent wild-mouse ecotropic virus CasBrE inserted. Antibodies against envelope were also effective against F-MuLV or F-MuLV-infected cells when transferred postchallenge, but then required an Fc portion to be protective. A reduction in the frequency of F-MuLV-producing cells in leukemic spleen could be induced by neutralizing mAbs (IgG2a) which mediated CMC but not others (Britt and Chesebro, 1983). Passive transfer of neutralizing antibodies against F-MuLV envelope furthermore protected against F-MuLV pathogenesis as a whole antibody but not as $F(ab')_2$ fragments (Collins *et al.,* 1983).

Friend virus has been used as a model to study the mechanisms by which immunization with live attenuated retroviruses protect against subsequent challenge with more virulent strains. The model used is complex but interesting, as

it is one of the few models of an immunosuppressive retrovirus inducing disease in immunocompetent mice. Challenge is performed with a combination of two viruses: a nonpathogenic replication-competent helper virus (F-MuLV), which contains all the required immunological determinants, and a replication-defective virus, spleen focus-forming virus (SFFV), required for pathogenicity in adult mice (Dittmer *et al.,* 1998). Immunization with attenuated F-MuLV elicits highly protective immune responses. It has been shown that this protective immune response involves a $CD4^+$ and $CD8^+$ T cell as well as a B cell component, as protective responses could only be transferred to naïve mice by combining all three cell populations in the transplant (Dittmer *et al.,* 1999a). Neutralizing antibodies are present in this response at a level below the concentration required to provide sterile immunity, but nevertheless appear to play an important role in the observed protection against disease, as evidenced by the following. First, protection from plasma viremia is completely correlated with the presence of F-MuLV neutralizing serum antibodies (Dittmer *et al.,* 1999a). Second, passive transfer of a F-MuLV neutralizing antibody to the same level as observed after immunization protects against pathogenicity, as all animals receiving the antibody recovered from Friend virus-induced disease (Dittmer *et al.,* 1999a). Third, complete protection correlated with the presence of both virus-neutralizing antibodies as well as primed cytotoxic T lymphocytes (CTL). Development of protection against persistent infection, in particular, only developed postimmunization after the appearance of neutralizing antibodies (Dittmer *et al.,* 1999b). This series of experiments indicates that neutralizing antibodies below the level required to induce sterile protection can play a critical role in protection against a retrovirus.

C. Rhabdoviruses—Vesicular Stomatitis Virus

A classical early study was that of Lefrancois (1984) who described a panel of mAbs to glycoprotein G of VSV. Neutralizing mAbs completely protected mice at relatively high doses. Nonneutralizing mAbs also protected, although they were less effective. Neither class of mAbs protected, if given as early as 2 hr postinfection. The nonneutralizing mAbs bound much less effectively than neutralizing mAbs to intact virions. However, the nonneutralizing mAbs could lyse infected cells in the presence of complement. Further, the $F(ab')_2$ fragment of a nonneutralizing mAb was not protective, whereas the same fragment of a neutralizing mAb was protective. It was suggested that the nonneutralizing mAbs were likely mediating protection via Fc-dependent effector function activity against infected cells. The behavior of the $F(ab')_2$ fragment of the neutralizing mAb suggests that effector systems are not crucial in the protective activity of this antibody. A very interesting feature of this study is the evidence it provides that a certain form of the envelope protein is present at the surface of infected cells, which is essentially absent from virions.

One of the most detailed comparisons of neutralization and protection is that of Bachmann *et al.* (1997) who described the properties of a large panel of mAbs to a single antigenic site on VSV-G. The authors reported a good correlation between neutralizing ability of antibody and affinity (avidity) for intact virus particles. However, these two parameters were reported not to correlate with the ability of antibody to protect SCID mice from viral challenge. Protection was said to depend simply upon a minimum serum concentration of antibody independent of affinity. However, most of the antibodies studied fell in a rather narrow affinity range ($2–9 \times 10^9$ M^{-1}; corresponding to a free energy of binding range of only about 1 kcal/mole), and indeed the minimum serum concentration for complete protection (3/3 mice) occurred over a corresponding narrow range of roughly 20–50 μg/ml. An antibody of significantly lower affinity (2.5×10^7 M^{-1}) was found not to protect mice at any of the concentrations used. This contradicted the thesis that neutralization and protection were not correlated, and was interpreted as a "threshold effect" below which the protective behavior of antibody was predictable by the law of mass action. One antibody of somewhat lower affinity (2×10^8 M^{-1}) but apparently disproportionately poor neutralizing ability was able to protect all challenged mice at approximately 20 μg/ml. In our view, this latter antibody is the only one that potentially offers a significant challenge to the correlation between affinity/neutralization and protection.

D. Alphaviruses

Early studies investigated mAbs to the envelope E1 and E2 proteins of Sindbis virus, Semliki Forest virus (SFV), and Venezuelan equine encephalomyelitis (VEE) virus. In a study on Sindbis virus (Schmaljohn *et al.,* 1982), neutralizing anti-E2 and nonneutralizing E1 antibodies were protective. The latter were shown to bind to infected cells but not to virions, and it was suggested that complement-mediated lysis of infected cells might be crucial. Later studies (Schmaljohn *et al.,* 1983) confirmed that the protective epitopes on E1 were only present on infected cell surfaces and not on virions.

Neutralizing and nonneutralizing mAbs against the E2 protein of SFV were shown to protect mice from lethal infection, although the former were effective at much lower doses (Boere *et al.,* 1985). Protection was observed for neutralizing mAbs at concentrations expected to saturate the challenge virus, and there was a good correlation between the efficacy of mAb with respect to *in vitro* neutralization and protection. Both neutralizing and nonneutralizing mAbs bound to infected cells and promoted complement-mediated lysis, although the neutralizing mAbs were more effective. Removal of the Fc part of a neutralizing mAb reduced its neutralizing capacity about 100-fold with a roughly comparable reduction in protective efficacy. Removal of the Fc part of a nonneutralizing mAb caused a complete loss of its protective ability. The results are

consistent with a crucial role for Fc-mediated effector functions in protection by the nonneutralizing antibody but a lesser role for the neutralizing antibody.

A potent anti-E2 neutralizing mAb was found to protect mice against lethal challenge with VEE (Mathews *et al.,* 1985). However, the corresponding $F(ab')_2$ fragment was not protective, although its neutralizing potency was the same as the parent mAb. The importance of Fc effector functions for protection was inferred, although the much shorter half-life of $F(ab')_2$ fragments *in vivo* may have contributed to its inefficiency. A nonneutralizing anti-E1 mAb was found to protect at much higher concentrations, presumably through its binding to infected cells, since the antibody did not bind to intact virions.

E. Arenaviruses

Passively administered immune serum, maternal antibodies, and anti-envelope glycoprotein (G1) mAbs have been shown to protect mice against lethal challenge with lymphocytic choriomeningitis virus (LCMV) (Baldridge and Buchmeier, 1992). Protection with mAbs required concentrations leading to essentially complete neutralization of virus *in vitro*. It appears that complete protection required antibody activity not only against free virions but also against infected cells. Thus $F(ab')_2$ fragments of a protective mAb retained neutralizing activity *in vitro* but were not protective *in vivo*. Furthermore, an IgG1 anti-GP1 mAb with more potent neutralizing activity than protective anti-GP1 IgG2a antibodies was not protective. The activity against infected cells did not involve complement, since C5-deficient mice were also protected by the IgG2a mAbs.

An interesting advantage of passively transferred neutralizing antibodies in LCMV infection is that, by effectively reducing the viral challenge, they can attenuate the subsequent CTL response (Wright and Buchmeier, 1991). This can be crucial in modulating the potentially destructive effects of an "over-vigorous" T cell-mediated immune response.

F. Coronaviruses

Several early studies looked at the ability of mAbs to protect mice against lethal challenge with murine hepatitis viruses (MHVs). The ability of a panel of 13 mAbs given i.p. to protect mice against intracerebral challenge with MHV-4 showed that 3 potently neutralizing mAbs specific for the E2 glycoprotein were protective at high dose (Buchmeier *et al.,* 1984). MHV-4 infection was not blocked completely, but viral loads in liver, brain, and central nervous system (CNS) were lower than in unprotected mice, and demyelination rather than fatal encephalomyelitis resulted. One potently neutralizing anti-E2 mAb was not protective. However, this mAb recognizes an epitope which is subject to rapid neutralization escape (Dalziel *et al.,* 1986), and the challenge virus may have contained escape variants of unusual fitness *in vivo* (M. J. Buchmeier,

personal communication). Nonneutralizing anti-E1 membrane glycoprotein and anti-N nucleoprotein mAbs were nonprotective. Another study investigated the activities of 20 mAbs specific for the E2 protein of MHV-4 (Wege *et al.,* 1984). Three mAbs that potently neutralized virus, inhibited cell fusion, and bound well to infected cells protected against intracerebral challenge. All other mAbs including some that neutralized more weakly and some that did not neutralize but bound well to infected cells were not protective. A further study assessed the activities of 4 anti-E1 mAbs. Only one was neutralizing and then only in the presence of complement. This mAb was protective. Of the 3 nonneutralizing mAbs, 1 was protective. The neutralizing mAb was equally effective in C5-deficient or decomplemented mice, suggesting that complement was not crucial for protection.

Monitoring of the viral load in liver, spleen, and brain allowed the effects of mAbs in i.v. challenge of mice with MHV-2 to be explored quantitatively (Nakanaga *et al.,* 1986). Protection against lethal challenge was provided by a potent neutralizing anti-E2 mAb, a nonneutralizing anti-E2 mAb, and a nonneutralizing anti-nucleoprotein (NP) mAb. However, the neutralizing mAb resulted in much lower viral loads in the tissues of infected animals, particularly in the brain where viral titers were 3–4 orders of magnitude lower for the neutralizing mAb-treated than the nonneutralizing mAb-treated mice. Furthermore, the neutralizing mAb was able to protect against a challenge dose of 30,000 pfu compared to the usual dose of 30 pfu. Protection by the anti-NP is presumably mediated by activity against infected cells that express NP on the surface, and this was confirmed for MHV-3 challenge (Lecomte *et al.,* 1987).

A comparison of a potent neutralizing IgG and the corresponding $F(ab')_2$ and Fab fragments showed that all three molecules could neutralize the neurotropic MHV-A59 virus, and all three could protect mice against lethal intracerebral challenge at very high doses (producing serum titers approximately 10^4 times 90% neutralization titers *in vitro*). The efficiency of protection of a given molecule correlated well with its potency of neutralization and affinity for antigen (Lamarre and Talbot, 1995). A single-chain Fv fragment of the same mAb could neutralize virus but was ineffective at protection, presumably because of its extremely short *in vivo* half-life (6 min) (Lamarre *et al.,* 1997).

G. Filoviruses

A recent study (Wilson *et al.,* 2000) describes the *in vitro* and *in vivo* effects of a panel of mAbs to the envelope glycoprotein of Ebola virus. The majority of mAbs protected mice against challenge with a mouse-adapted Ebola virus. None of the protective antibodies neutralized the virus in a plaque assay in the absence of complement. In the presence of complement, a subset of antibodies neutralized the virus. For these antibodies, complete protection was observed at a serum concentration approximately an order of magnitude greater than the

concentration required for 80% neutralization *in vitro*. A caveat to note here is that neutralization is carried out on plaquing virus, although most infectious particles are nonplaquing, as revealed by the correspondence of the challenge dose of $300LD_{50}$ to only 10 pfu of virus.

Using a potent human neutralizing anti-Ebola antibody (Parren *et al.,* 2001a), we found that protection against a guinea pig-adapted virus in a guinea pig model required serum antibody concentrations corresponding to essentially complete virus neutralization *in vitro* (Maruyama *et al.,* 1999; Parren *et al.,* 2001a). In this case, the viral challenge was much higher (10^4 pfu; $>10^5$ LD_{50}) than the previous example (10 pfu; 300 LD_{50}).

H. Flaviviruses

Early studies showed that neutralizing mAbs against the envelope glycoprotein and nonneutralizing antibodies against the nonstructural glycoprotein of yellow fever (YF) virus could protect mice against lethal intracerebral challenge (Gould *et al.,* 1986; Schlesinger and Chapman, 1995). Neutralizing mAbs were more effective and could protect against more neurovirulent strains of YF virus, against which the anti-NS mAbs were ineffective. More recently, it was shown that the $F(ab')_2$ fragments of a strongly neutralizing IgG2a mAb were equally effective at neutralization but did not mediate protection *in vivo* (Schlesinger and Chapman, 1995). However, incubation of $F(ab')_2$ virus complexes with a rabbit IgG anti-mouse IgG resulted in protection. In contrast, the corresponding rabbit $F(ab')_2$ fragment did not afford protection. In addition, an IgG1/IgG2a switch variant was suggested to have the specificity of the parent IgG1, but the Fc of IgG2a was more effective in protection than the parent IgG1. The results imply a role for Fc-mediated effector systems in protection against YF.

A recent study (Forthal *et al.,* 1993) on the development of humoral immunity to tick-borne encephalitis virus (TBEV) provides some novel insight into how antibodies to virions and to infected cells can be important. Passive transfer of polyclonal neutralizing antibodies at doses expected to completely neutralize virus *in vitro* protected mice from lethal TBEV challenge. However, despite the high doses used, protection was not sterile and some viral replication occurred. Transfer of sera from these mice could confer complete protection from disease on naïve mice. Interestingly, transfer of T-enriched spleen cells from the mice could not confer protection on naïve mice. The specificity of the transferred antibodies was shown to be for the nonstructural protein (NS1) which is absent from the virion, implying that antibody activity against infected cells can confer protection.

Antibody-dependent enhancement (ADE) of TBEV can be demonstrated *in vitro,* and it has been suggested that postexposure Ig prophylaxis, which is widely used in nonvaccinated individuals who are bitten by ticks in areas where the virus is endemic, might worsen the outcome of infection. However, studies in

mice with antibodies that did show ADE *in vitro* gave no evidence of enhanced infection *in vivo* under a range of conditions (Kreil and Eibl, 1997).

It should be noted that an *in vivo* relevance of antibody-mediated enhancement of infection is uncertain in many cases. The flavivirus dengue virus, however, forms a clear example of a virus for which a strong *in vivo* enhancement by antibodies against envelope glycoprotein has been demonstrated (Halstead, 1979, 1982).

I. Herpesviruses

Passive transfer of immune rabbit anti-IgG was shown to protect mice against lethal challenge with HSV type 1 (HSV-1) after corneal inoculation and HSV-2 after subcutaneous inoculation (Oakes and Lausch, 1981). Protective serum neutralizing titers were approximately 1:50. The corresponding $F(ab')_2$ preparation was equally effective at neutralization *in vitro* and protection *in vivo*. However, interestingly, only the IgG preparation showed activity in established infection. In another model of HSV-1 infection, mice were inoculated with virus in the footpad and then treated 4 hr later with a single dose of immune rabbit IgG or five daily doses of the corresponding $F(ab')_2$ preparation (McKendall, 1985). Both treatments led to serum neutralizing antibody titers of 1:16. IgG treatment markedly, and $F(ab')_2$ treatment moderately, reduced footpad viral titer, viral spread to sciatic nerve and spinal cord, and the establishment of latency. The IgG effects were also apparent in C5-deficient mice. A further study looked at the activity of immune mouse and human IgG against HSV in nude mice (Hayashida *et al.,* 1982). Relatively high concentrations of antibody (1 ml of serum neutralizing titer $>1:32$ given i.p.) were required to inhibit the development of skin lesions and to prolong survival of lethally infected mice. Purified $F(ab')_2$ fragments were ineffective even when given repeatedly to maintain neutralizing titer. IgG was effective in C5-deficient mice. The results are consistent with the notion that ADCC becomes crucial in protection against HSV if sterile protection is not provided at challenge.

Many studies have looked at the ability of mAbs to protect against HSV infection. Early papers showed that both neutralizing anti-gD and gC mAbs and nonneutralizing anti-gD, gC, gB, gD, and gE mAbs could protect against lethal footpad challenge in mice (Balachandran *et al.,* 1982; Dix *et al.,* 1981). The latter study found that a mixture of mAbs had an efficacy corresponding roughly to the sum of that of the individual mAbs and that protection was equally apparent in C5-deficient mice. A good correlation was reported between protection and the titers of the nonneutralizing mAbs in an ADCC assay. It was suggested that ADCC could provide protection against lethal HSV infection.

In the murine zosteriform spread model, infection is initiated at a cutaneous site and spreads to the peripheral nervous system, from which the virus (HSV-1) reemerges and infects regions of the epithelium remote from the inoculation

site. The model mimics the course of human herpetic disease. High doses (0.2–5 mg/mouse) of anti-gD, gC, and one gB mAb offered partial protection in the model (Mester *et al.,* 1991). It was reported that protection correlated better with *in vitro* ADCC than neutralization activity. In addition, $F(ab')_2$ and Fab fragments of two protective anti-gC mAbs were nonprotective.

Investigation of the ability of a panel of anti-gD mAbs to protect against HSV-2 challenge showed that a mAb, neutralizing virus even in the absence of complement, was the most effective (Ishizaka *et al.,* 1995). Of a series of mAbs that neutralized in the presence of complement, the order of efficacy of a series of switch variants was IgG2a>IgG2b>IgG1. A human neutralizing anti-gD mAb was shown to completely protect nude mice against lethal challenge with HSV-1 using a high iv dose (450 μg/mouse) (Sanna *et al.,* 1996). The same mAb applied topically could protect C57Bl/6 mice against vaginal challenge with much lower doses of mAb (400 ng/mouse) (Zeitlin *et al.,* 1996). IgG and $F(ab')_2$ fragment were approximately equivalent in topical protective ability, with the Fab fragment being somewhat less effective.

J. Orthomyxoviruses—Influenza Virus

A detailed study (Mozdzanowska *et al.,* 1997) has compared the *in vitro* neutralization and *in vivo* protective activities of a number of mAbs to influenza virus HA. The mAbs were chosen to have a range of neutralizing activities. In the presence of complement (1.6% serum), neutralizing ability was differentially enhanced from 2- to 75-fold. The serum antibody concentration conferring protection was two to three orders of magnitude higher than the concentration required for 50% neutralization *in vitro* in the presence of complement. However, the rank order of protective ability was not well predicted by the order of neutralizing ability.

K. Picornaviruses

McCullough *et al*. (1986) have shown in passive transfer studies that a good correlation exists between neutralization and protection against FDMV, as only antibodies which neutralized a certain FDMV challenge isolate strongly or moderately provided protection. Interestingly, however, these antibodies protected at concentrations 10- to 60-fold below those required for *in vitro* neutralization of FDMV. The enhanced protective ability of these antibodies appeared Fc-mediated, as conversion of the mAb into $F(ab')_2$ fragments reduced protective ability, whereas their neutralizing ability was mostly retained (McCullough *et al.,* 1986). Neutralization titers were not affected by the addition of complement, suggesting that CMC did not play a significant role (McCullough *et al.,* 1988). The effect rather appeared due to the induction of phagocytosis of sensitized virus, as impairment of phagocytosis by silica treatment abrogated the enhanced protection. In silica-treated mice, neutralizing antibody concentrations

well over the 90% neutralization titers were required for protection, indicating that in the absence of phagocytosis, protection correlates with neutralization of all the challenge virus. It is likely that efficient protection against FDMV infection by neutralizing antibody at low occupancy is provided by an efficient phagocytosis of sensitized virions, as has been demonstrated for FDMV *in vitro* (McCullough *et al.,* 1988, 1992).

L. Paramyxoviruses

Many studies have looked at the activities of antibodies against RSV, and one anti-RSV mAb is in clinical use for the prophylaxis of RSV disease. Passively transferred polyclonal antibody administered i.p. to produce a serum neutralizing titer of 1:380 or greater provided sterile protection in the lungs of cotton rats challenged with RSV (Prince *et al.,* 1985b). Similarly, high doses of anti-F or anti-G glycoprotein mAbs reduced the virus titers in the lungs of cotton rats challenged with RSV to undetectable levels (Walsh *et al.,* 1984). From 15 anti-RSV mAbs, including 10 to the F glycoprotein, only 2 were neutralizing *in vitro,* and these were the only mAbs that were completely protective in mice (Stott *et al.,* 1984). In contrast, a nonneutralizing anti-G mAb that protects SCID mice against intranasal RSV challenge at a dose of 5 mg/kg has been described (Corbeil *et al.,* 1996). Protection is reduced in decomplemented or C5-deficient mice, suggesting complement is important in the antiviral activity. In bovine RSV, two neutralizing anti-F mAbs administered by the intratracheal route at moderate dose (0.4 mg/kg) protected against infection, whereas a nonneutralizing mAb was not protective (Thomas *et al.,* 1998). An anti-F IgA at 0.5 mg/kg introduced intranasally to mice prior to RSV challenge greatly reduces virus titers in the lungs (Weltzin *et al.,* 1994). In human phase III trials, the enhanced activity of one mAb relative to another has been attributed at least in part to its better neutralizing ability *in vitro* (Johnson *et al.,* 1999).

The antiviral activity of a panel of nine mAbs to the F protein of Sendai virus has been compared *in vitro* and *in vivo* (Mochizuki *et al.,* 1990). None of the mAbs were neutralizing in standard assays, but two were potently neutralizing in the presence of complement. These mAbs protected very young mice against lethal infection and allowed them to thrive in terms of body weight gain, when given at high dose. Three other mAbs showing weak or no neutralization in the presence of complement were also protective. Two of these mAbs were of the IgG1 isotype, which lead to the suggestion that ADCC rather than complement might be important in protection.

M. Reoviruses

Passive transfer of a neutralizing mAb against bluetongue virus (BTV) has been shown to protect mice and sheep from disease, whereas nonneutralizing mAb did not protect. A neutralizing antibody titer of 1:20 protected the

sheep from disease, but did not provide sterile immunity, as an increase in neutralization titers was observed 8 to 9 days postchallenge. The results are in strong contrast with the inability of 100-fold higher neutralizing antibody titers to clear virus from sheep with existing infection (Letchworth and Appleton, 1983). The impact of antibody on established infection is discussed in detail below.

N. Neuropathogenic Lactate Dehydrogenase-Elevating Virus and HIV-1: Cautionary Notes on the Difficulties of Comparing in Vitro and in Vivo Antibody Activities

Infection of mice of certain strains with neuropathogenic LDV results in a fatal paralytic disease through interaction with endogenous retroviruses. The infection of anterior horn neurons by LDV and the development of disease are prevented by anti-LDV antibodies. The mechanism by which motor neurons were protected from infection by LDV was unclear as in addition to neutralizing antibodies also nonneutralizing (polyclonal) antibodies prevented neuron destruction and disease. In addition, protection occurred in the absence of any apparent effect of antibody on LDV replication in a subpopulation of macrophages known to be the primary permissive host cells. The resolution to this paradox is a lesson with regard to the ability of viral quasispecies to mislead (Chen *et al.,* 1999).

It appears that neuropathogenic LDV isolates contain both neuropathogenic and nonneuropathogenic quasispecies. Using biological clones, it was shown that the nonneuropathogenic species were about 100 times more resistant to *in vitro* neutralization than the neuropathogenic species. Some antibodies therefore do not neutralize nonneuropathogenic viruses and are scored as "nonneutralizing" by *in vitro* assays. These antibodies do, however, neutralize neuropathogenic viruses and therefore are protective *in vivo*. The paradox described above appears to be due to LDV heterogeneity. It is of interest that mixed virus populations with distinct neutralization properties have also been described for another arterivirus: PRRSV (Weiland *et al.,* 1999).

T-cell line adapted strains of HIV-1 represent a striking example of how studying neutralization of viral variants selected *in vitro* may lead to aberrant conclusions with respect to the neutralizing responses in infection. The adaptation of HIV-1 to growth in $CD4^+$ T cell lines selects for variants that are readily neutralized by soluble CD4 and a large spectrum of different mAbs. By contrast, plasma virus or viruses which have only been passaged in primary cultures of activated peripheral blood mononuclear cells (PBMC) are mostly resistant to neutralization by these same ligands (reviewed in Moore and Ho, 1995; Parren *et al.,* 1999). An explanation for this phenomenon may be the high expression levels of heparan sulfate proteoglycans on the surface of T cell lines, which through an interaction with gp120 may select for HIV-1 viruses with unusual

strongly basic gp120 V3 loops (Moulard *et al.,* 2000). The envelope spike of these TCLA viruses adopts a much more open configuration, which provides access to a range of epitopes which are inaccessible on naturally occurring HIV-1 isolates. Consequently, sera from HIV-1 infected individuals, as well as sera from vaccinees immunized with recombinant HV-1 envelope subunits, typically contain high titers of neutralizing antibodies against TCLA strains of HIV-1, whereas the more relevant titers against HIV-1 primary isolates are usually very poor.

O. Further Cautionary Notes on Comparing in Vitro and in Vivo Antibody Activities: the Possible Effects of Virus Challenge Dose and Anatomical Considerations

It is in the nature of passive transfer studies that typical virus challenge doses are relatively large to ensure that all control animals become infected. It is sometimes argued that, in some human infections, challenge doses could be smaller and protection achieved at lower antibody concentrations than indicated from animal studies. For example, SIV or SHIV experiments in macaques usually involve virus challenge doses of 10 AID_{50} (50% animal infectious dose) or more. However, the typical human challenge dose with HIV-1 is probably 0.01 AID_{50} or less, since the frequency of infection is about 1:100 or less, depending upon the nature of exposure. This has been interpreted to indicate that relatively low vaccine-induced serum antibody concentrations compared to *in vitro* neutralization titers may offer protective benefit against HIV-1 infection. From a thermodynamic standpoint, this argument appears to have little merit, since the amount of serum antibody will be in vast molar excess over the challenge virus in most scenarios, and the extent of antibody coating of virus (and therefore neutralization) should be determined by the binding constant of antibody to virus rather than the number of challenge particles. However, there are no studies of which we are aware that have directly addressed the ability of antibody to protect at very low challenge doses. For a given virus, the existence of factors during low dose natural infection processes outside the laboratory that complicate the extrapolation from animal studies to humans cannot be categorically excluded.

Anatomical considerations may also complicate the interpretation of *in vivo* protection data. Antibody serum concentrations can be readily measured. For the most part, tissue antibody concentrations are not measured, and the concentration of antibody that may in fact be responsible for blocking infection at a tissue site is not known. It is possible that the high serum antibody concentrations required for protection in some instances reflect the difficulty of achieving protective antibody concentrations at a critical tissue site. If some antibodies are better able than others to diffuse to tissue sites, they may therefore show enhanced *in vivo* relative to *in vitro* activity.

VIII. Mechanisms of Antiviral Antibody Activity in Established Infection

Although there is evidence that antibody can impact upon a number of established viral infections (Chanock *et al.,* 1993), there are little quantitative data relating *in vivo* and *in vitro* activities. In the data reviewed below, we consider antibody given 1 day or more after virus as having activity against "established" infection as opposed to the prophylactic activity considered above. This is clearly a somewhat artificial distinction, and antibody generally appears to have diminished activity the further infection progresses.

Elegant studies show that SCID mice infected with influenza A for 1 day can be cured using neutralizing mAbs to viral HA with a close correlation between prophylactic and curative activity (Mozdzanowska *et al.,* 1997). The data suggest that the curative effect is mainly due to the neutralizing activity of antibody against free virus, with some contribution from activity against infected cells. This conclusion is supported by investigation of two nonneutralizing mAbs, one to viral NA and one to M2 (Mozdzanowska *et al.,* 1999). Both mAbs reduced pulmonary viral titers in established infection by 100- to 1000-fold, but they failed to clear infection even at high dose in combination.

It might be predicted that neutralizing antibody would have activity against a virus such as influenza that does not propagate via cell-to-cell spread and is cytopathic for infected cells. The presence of high levels of neutralizing antibody should eventually terminate infection. However, for viruses that do propagate via cell-to-cell spread, antibody would be expected to be less effective, since higher concentrations of neutralizing antibody are generally required to inhibit infection by this route than are required to inhibit infection by free virions (Hooks *et al.,* 1976; Pantaleo *et al.,* 1995). Indeed, for HIV-1, hu-PBL-SCID mouse studies suggest that the virus replicates unhindered in a significant proportion of cases in the presence of serum concentrations of a single mAb that are largely protective if administered prior to virus challenge (Poignard *et al.,* 1999). In the remainder of cases, neutralization escape occurs rapidly, showing that the mAb does exert some pressure on virus replication in those animals. If a cocktail of mAbs is administered at high dose (50 mg/kg) in established infection, then neutralization escape is rapidly apparent. However, as the serum mAb concentrations wane to around 10 times 90% *in vitro* neutralization levels, the wild-type neutralization-sensitive virus reemerges. This suggests that the escape variants are less fit than the wild type, and establishes a high threshold of serum antibody to impact upon propagation of infection. Another study shows that passive transfer of neutralizing polyclonal anti-SIV antibody has a very modest effect upon SIV replication in macaques (Binley *et al.,* 2000).

The effect of antibody on established retrovirus infection has also been investigated in the F-MuLV system (Hasenkrug *et al.,* 1995). High doses of neutralizing mAb could induce recovery, but success was dependent upon the presence of

both $CD4^+$ and $CD8^+$ T lymphocytes. The results strikingly demonstrate the ability of antibody and two populations of T cells to work together in a way which is not fully understood at this time.

RSV is a virus that, from its name, one might expect to propagate via cell-to-cell spread. However, although syncytia are observed *in vitro,* they have not been observed *in vivo,* and the mode of infection propagation is unclear. Because the tracheal epithelium is only sparsely infected at any point in time, however, it is unlikely that cell-to-cell spread is a major mechanism of spread *in vivo* (McIntosh and Chanock, 1990). In any case, a number of studies have shown that neutralizing antibody can have a therapeutic effect. Early work in the cotton rat model showed that passively administered polyclonal immune antibody resulting in serum neutralizing titers in the range 1:400–1:1000 could drastically reduce the virus titers in the lungs by as much as a factor of 10^4 (Prince *et al.,* 1985a). Later work focused more on intranasal inoculation when relatively small quantities of neutralizing antibody were found to be effective in cotton rats, mice, and monkeys (Crowe *et al.,* 1994; Hemming *et al.,* 1985; Prince *et al.,* 1987, 1990; Weltzin *et al.,* 1996). Therapeutic effect could be achieved with $F(ab')_2$ (Prince *et al.,* 1990) and Fab fragments (Crowe *et al.,* 1994) of neutralizing antibody, suggesting that it was a direct result of interaction between antibody and virus. In the case of Fab fragments, viral loads in the lungs of infected mice could be reduced by a factor of almost 10^4 by as little as 13 μg of protein introduced intranasally.

A number of studies show that antibody can be highly effective against established CNS infection in rodent models, preventing disease or death. Administration of large amounts of hyperimmune anti-HSV-1 serum (0.5 ml/mouse) completely protected naïve animals from illness when given up to 24 hr following footpad challenge (Lubinski *et al.,* 1998). As the time interval from challenge to antibody administration was increased, the incidence of illness increased to 25% at 24 hr, 62% at 72 hr, and 86% at 96 hr. MAbs at high dose given 24 hr post-exposure were similarly shown to protect against HSV-1-induced ocular disease in mice, with protection occurring for nonneutralizing as well as neutralizing mAbs, suggesting, as expected, the importance of activity against infected cells. Protection against intracerebral challenge of mice with YF virus was shown to occur even when mAbs were given i.p. several (3–5) days after virus inoculation when peak infectious virus titers and histopathological evidence of infection were present in brains. Nearly complete protection (eight of nine animals) was noted for one mAb given at a dose of approximately 1.5 mg/kg 4 days after cerebral challenge. Protection was apparent for neutralizing and nonneutralizing mAbs. Furthermore, some of the nonneutralizing mAbs were shown to inhibit viral replication *in vitro* in a neuroblastoma cell line, hinting at a novel mechanism of antibody protection.

A thorough study showed that both polyclonal antibodies and mAbs can protect against neurally spreading reovirus type 3 (Dearing) in mice (Virgin *et al.,* 1988) even when given several days after cerebral or footpad challenge. For instance, a potent neutralizing mAb given at approximately 8 mg/kg 1 week postchallenge led to the survival of roughly half of the animals infected. Protection was apparent with both neutralizing and nonneutralizing polyclonal antibodies, but a nonneutralizing mAb was far less effective than the neutralizing mAb. Serum complement was not required for antiviral activity. Attempts to investigate the activity of $F(ab')_2$ fragments in comparable experiments were thwarted because of the short half-life of $F(ab')_2$, so that even daily administrations did not maintain serum levels. This careful work emphasizes that, unless $F(ab')_2$ levels are specifically monitored, conclusions drawn from comparing IgG and $F(ab')_2$ should be treated with caution. Further studies show that high doses of neutralizing mAbs can protect mice against lethal challenge with Theiler's murine encephalomyelitis virus (Fujinami *et al.,* 1989) and neurotropic measles virus (Liebert *et al.,* 1990) when given 2 days and 5–8 hr postexposure, respectively. In the latter case, nonneutralizing mAbs are ineffective. Further, the latter study again provides evidence of the ability of antibody to restrict viral replication inside an infected cell by binding to viral antigen.

This is an interesting and potentially very important phenomenon. It was first described by Fujinami and Oldstone (1979, 1980) for measles virus-infected cells, and has been extensively investigated by Griffin and co-workers for Sindbis virus infection of neurons (Levine *et al.,* 1991). Treatment of persistently infected SCID mice with mAbs to the E2 glycoprotein of Sindbis virus results in the gradual noncytopathic removal of viral RNA by a process which is independent of complement and T cells. Antibodies can also clear Sindbis virus from persistently infected neuronal cell cultures. The isotype of antibody is not important, but bivalency is required (Ubol *et al.,* 1995). It is suggested that clearance involves a novel mechanism triggered when antibody cross-links viral protein expressed on the surface of infected cells. A similar mechanism has been proposed for antibody activity against rabies-infected neural cells (Dietzschold *et al.,* 1992). This mechanism is distinct from earlier studies that described the ability of antibody to modulate antigen expression at the surface of infected cells and thereby reduce the efficiency of virus budding (e.g., Chesebro *et al.,* 1979).

Finally, nonneutralizing IgA mAbs can resolve an ongoing rotavirus infection, apparently by interaction between antibody and virus during transcytosis (Burns *et al.,* 1996). SCID mice, infected with rotavirus for at least 2 months, were transplanted subcutaneously with hybridomas secreting mAbs to VP4 (an outer capsid viral protein) and VP6 (a major inner capsid viral protein). Only two nonneutralizing IgA mAbs to VP6 were capable of resolving chronic infection. These mAbs were not, however, active when presented directly to the luminal

side of the intestinal tract, suggesting their mode of action is during transcytosis as described *in vitro* (Bomsel *et al.,* 1998; Manzanec *et al.,* 1992). Neutralizing IgA mAbs to VP4 did not resolve infection.

IX. Observations with Nonviral Pathogens

We note that a correlation between inactivation of infectivity of a pathogen and antibody coating extends beyond the viruses on which we have focused in this review. For example, as discussed above, a correlation exists between neutralization of the intracellular bacterium *Chlamydia trachomatis* and coating of the infectious elementary bodies with antibody (Peeling and Brunham, 1991). Complement may enhance neutralization of opsonized *C. trachomatis* (Megran *et al.,* 1988). Passive transfer of an antibody against an outer surface protein of *Borrelia burgdorferi,* which strongly stained nonpermeabilized *B. burgdorferi* cells, completely protected mice from challenge (Mbow *et al.,* 1999). Protection against the parasite *Trypanosoma cruzi* by antibody, furthermore, has been correlated with antibodies that bind to living trypomastigotes in immunofluorescence (Heath *et al.,* 1990).

X. Conclusions

We began with an assertion that some general rules describing the *in vitro* and *in vivo* activities of antibodies against viruses can be discerned from the literature. We now review our interpretation of the data as a whole and its significance for these rules.

There has been much discussion of the mechanisms of neutralization of viruses *in vitro*. A prominent opinion has been that antibodies can act at many different stages of the infectious process, including post-viral entry to the target cell, that several different mechanisms may operate in concert, and that critical sites on the virion surface must be occupied for neutralization (Dimmock, 1993). We disagree. We believe that the data are consistent, in the vast majority of cases, with a simple occupancy model essentially as initially proposed by Macfarlane Burnet in 1937. According to this model, neutralization occurs when a sizable fraction of available sites on the virion are occupied by antibody, leading to inhibition of virus attachment or interference with the entry (fusion) process. The relatively large bulk of the antibody molecule, very roughly similar to that of a typical envelope spike for an enveloped virus, is suggested to be critical (Fig. 6).

The model is consistent with a number of observations. First, we have shown here that there is a roughly linear relationship between the surface area of a virus and the number of antibody molecules bound at neutralization. This number is approximately that predicted to effectively coat the virion particle, given the

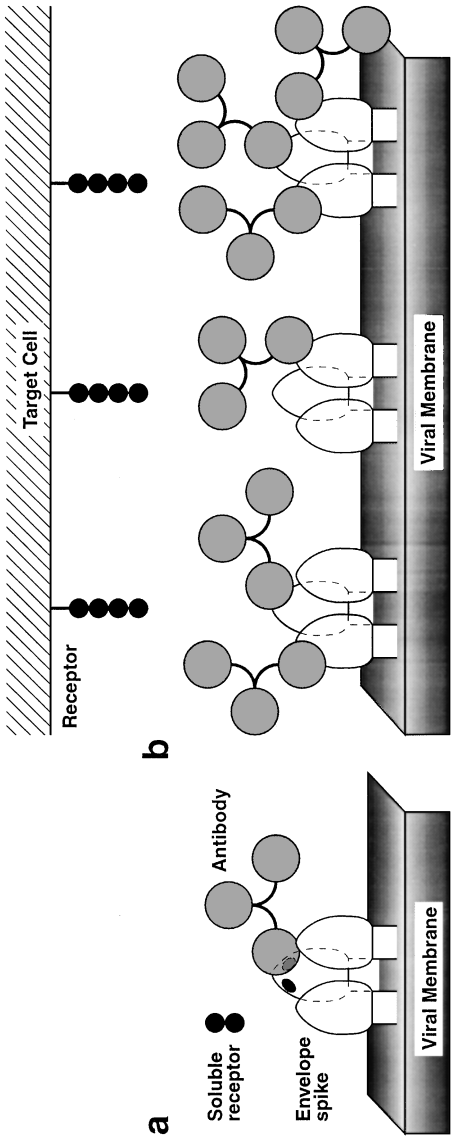

FIG. 6. Model for proposed interactions between envelope spikes and neutralizing IgG. The molecules depicted are drawn to scale assuming that an IgG molecule has roughly the same molecular weight as the monomer of a typical trimeric envelope spike. The model explains how antibody coating of the virus surface may interrupt infection without occupying all available binding sites. (a) Envelope spike with an IgG molecule bound. Additional binding sites on the envelope spike are still available and the binding of additional antibodies to distinct epitopes or recombinant soluble receptor molecules may not be inhibited. An antibody to the V3 loop on HIV-1 gp120, for example, will not inhibit binding of soluble CD4. (b) Envelope spike with various numbers of IgG molecules associated. Coating of virion spikes with antibody, irrespective of the epitope(s) recognized, interferes with the initiation of a productive infection, as the establishment of multiple critical contacts with membrane receptors required for infection is inhibited by steric hindrance and geometric constraints (see also Parren *et al.* (1998)).

size of the antibody molecule. Second, any antibody that binds well to and coats the virion surface should neutralize the virus, as is almost universally found. Nonneutralizing antibodies should not coat the virus, although they may bind at lower levels of occupancy. Heterogeneous envelope spikes provide an opportunity for binding to virions without neutralization because of low occupancy, as discussed above for rabies virus and the FPV/VSV mixed virions. Third, the model predicts that the precise epitope recognized by the antibody on the virion surface should not be crucial. Rather, the number of antibody molecules bound per unit area, which will be determined by the affinity of antibody for virion-expressed antigen, will be most important. This is precisely that which is found for HIV-1, as discussed above.

Antibody enhancement of viral infection *in vitro* receives much attention, although its significance *in vivo* has only been demonstrated for dengue virus infection. It appears to occur in the presence of subneutralizing concentrations of neutralizing antibodies. The model described above suggests that this is a phenomenon arising from low occupancy of virion sites, as discussed earlier.

The question whether some antibodies bind well to the virus without neutralization is often raised. In particular, the concern is expressed that nonneutralizing antibodies might compete with the binding of neutralizing antibodies to virus, thereby interfering with neutralization. First, we strongly question the evidence for binding but nonneutralizing antibodies. Generally, there is a very strong correlation between occupancy and neutralization, and most examples of such nonneutralizing antibodies can indeed be explained by poor virus binding, or even a failure to appreciate that antibodies which bind well to isolated envelope or capsid molecules do not necessarily bind well to the virus particle. Some convincing but isolated examples exist, such as the rabies mutant virus, which merit further investigation. Second, to our knowledge, there is no convincing and confirmed evidence for a nonneutralizing antibody interfering with neutralization. Virion coating but non-neutralizing antibodies therefore do not appear to play a significant role (if any) in the humoral response against viruses.

Antibody activity *in vivo* can arise through binding to virions or virion products on infected cells. Protection by antibody at the level of sterile immunity requires activity against free virions, i.e., neutralizing antibodies. Several studies with mAbs show that sterile immunity is only achieved when serum concentrations of the challenged animal are of the order of two to three orders of magnitude higher than *in vitro* neutralization titers, that is, serum concentrations capable of neutralizing essentially all of the challenge virus. Expressed in another way, serum neutralizing titers of 1:100–1:1000 are required for sterile protection. In a number of cases (discussed above), even such high levels of neutralizing antibody do not provide sterile protection, although they do prevent disease. Most vaccines do not elicit very high neutralizing titers, especially over an extended period, but then they probably do not provide sterile protection. Rather, it seems

likely that they reduce the effective challenge to such a level that infected cells can be controlled by cellular immunity and possibly by antibodies.

There are a number of cases (discussed above) where *in vivo* protection by neutralizing antibodies is independent of the presence of the Fc part of the molecule. In these cases, it seems likely that the mechanism of protection *in vivo* is essentially equivalent to neutralization *in vitro*. In a number of other cases, it appears that $F(ab')_2$ fragments, that are equally active as whole IgG molecules at neutralization *in vitro,* are ineffective at protection. Further, neutralizing mouse IgG1 switch variants are ineffective at protection, whereas IgG2a molecules are effective. In many of these examples, protection is independent of complement, suggesting that protection by neutralizing antibodies requires activity against infected cells as well as free virions. This requirement, in some cases, for activity against infected cells as well as virions may contribute to observations of incomplete correlation between neutralization and protection for neutralizing antibodies, although other factors such as the use of different cell types *in vitro* and *in vivo* should be considered. It is worth emphasizing, though, that most neutralizing antibodies protect at appropriate concentrations, and antibodies assessed as the most potent in neutralization assays *in vitro* generally are the most effective at protection *in vivo*.

There are numerous examples of protective activity exhibited by nonneutralizing antibodies. This activity appears to be directed at infected cells, and generally appears to be somewhat less potent than that of neutralizing antibodies. For instance, cases are described above where neutralizing antibodies are protective against higher challenge doses or more pathogenic viruses than nonneutralizing antibodies. In many cases, protection by nonneutralizing antibodies is shown to depend critically on the Fc part of the antibody molecule and to occur in complement-deficient mice, suggesting that ADCC (or phagocytosis) may be crucial in clearing antibody-complexed infected cells. It should be noted that protection with nonneutralizing antibodies is mostly restricted to protection against enveloped viruses.

What is the significance of these conclusions for vaccine design? In the first case, the time-honored focus on eliciting neutralizing antibodies is well justified. Serum neutralizing antibody titers of the order of, or greater than, 1:100 provide the greatest likelihood that antibody alone can protect against viral challenge. In many cases, antibody and cellular responses may cooperate to protect, although, with notable exceptions (Dittmer *et al.,* 1999a), this is an underexplored area.

The model described above asserts that neutralization is determined by the extent of coating of virus by antibody. At a given antibody concentration, this is in turn determined by the affinity of antibody for the antigen on the virion surface. Hence, the model predicts that a vaccine should simply aim to elicit antibodies of the highest affinity for the virion surface antigen. This is most directly achieved by immunization with molecules identical to or as similar to the viral surface

antigen as possible. This may not be easy. We have argued that a number of enveloped viruses have evolved surface proteins of low immunogenicity (Burton and Parren, 2000). Subunit envelope proteins appear, in a number of instances, to elicit antibody responses that show little reactivity with the form of the envelope (usually oligomeric) expressed on the virion surface (Parren *et al.,* 1998; Roben *et al.,* 1994; Sakurai *et al.,* 1999; Sattentau and Moore, 1995).

Another interesting question is the value of eliciting antibodies targeted to infected cells rather than virions. There are examples, such as arenaviruses, where complete passive protection appears to require antibodies to infected cells as well as virions. Of course, neutralizing antibodies could fulfill both roles by binding, for instance, to envelope molecules on virions and infected cells. However, it is also possible that epitopes expressed on virions are not expressed optimally on infected cells, for ADCC for example, and then the induction of nonneutralizing antibodies may be beneficial. This is a factor worthy of consideration in vaccine design, particularly using subunit proteins to elicit antibodies.

Finally, could passively administered antibodies be used in the treatment of acute viral diseases? The studies discussed above suggest high doses would be required to have any reasonable chance of efficacy. However, given the availability of human antibodies from new technologies such as transgenic mice and phage display (Burton and Barbas, 1994; Green *et al.,* 1994; Lonberg *et al.,* 1994; Winter *et al.,* 1994), and the ability to produce large amounts of such antibodies relatively cheaply in culture systems (e.g., Verma *et al.,* 1998), larger animals (e.g., Pollock *et al.,* 1999), or plants (e.g., Fischer *et al.,* 1999), antibody intervention in acute viral disease may become increasingly realistic. Antibodies are already used in certain situations in a postexposure mode to prevent, e.g., disease due to Junin virus (Argentine hemorrhagic fever), rabies virus, and TBEV. However, the evaluation of antibodies in humans following the appearance of symptoms in infections due to viruses such as RSV, dengue, and hanta would be of great interest.

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