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Review Article

Neutralization of Virus Infectivity by Antibodies: Old Problems in New Perspectives

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Neutralizing antibodies (NAbs) can be both sufficient and necessary for protection against viral infections, although they sometimes act in concert with cellular immunity. Successful vaccines against viruses induce NAbs but vaccine candidates against some major viral pathogens, including HIV-1, have failed to induce potent and effective such responses. Theories of how antibodies neutralize virus infectivity have been formulated and experimentally tested since the 1930s; and controversies about the mechanistic and quantitative bases for neutralization have continually arisen. Soluble versions of native oligomeric viral proteins that mimic the functional targets of neutralizing antibodies now allow the measurement of the relevant affinities of NAbs. Thereby the neutralizing occupancies on virions can be estimated and related to the potency of the NAbs. Furthermore, the kinetics and stoichiometry of NAb binding can be compared with neutralizing efficacy. Recently, the fundamental discovery that the intracellular factor TRIM21 determines the degree of neutralization of adenovirus has provided new mechanistic and quantitative insights. Since TRIM21 resides in the cytoplasm, it would not affect the neutralization of enveloped viruses, but its range of activity against naked viruses will be important to uncover. These developments bring together the old problems of virus neutralization—mechanism, stoichiometry, kinetics, and efficacy—from surprising new angles.

1. Introduction

Neutralizing antibodies (NAbs) are the best correlate of protection from viral infection after vaccination [1–8]. Likewise, they are markers of immunity against reinfection after an acute infection has been cleared. Such immunity can be lifelong [9–11]. Many vaccines against viral infections are good inducers of protective neutralizing antibody responses, but recalcitrant problems remain in the field of viral vaccination. One problem is antigenic variability. The antigenic targets for neutralizing antibodies on influenza virus vary abundantly, and therefore a new vaccine must be prepared every new season [7]. Hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are even more variable, and after years of research we still cannot induce immune responses that protect against them [7, 12–14].

Antibodies are the products of the adaptive humoral immune response; the molecules they recognize are called antigens; the molecules that elicit the antibody response are immunogens. Hence some proteins, particularly foreign ones, are both immunogens, inducing the production of specific antibodies against themselves, and antigens, the targets of the response. Other molecules, for example, small organic compounds, may bind with high specificity to antibodies without being able to elicit those antibodies except when conjugated to larger carriers [15]. Such small molecules are called haptens. They illustrate the important distinction between immunogenicity, the capacity to elicit an immune response, and antigenicity, the capacity to be recognized by the immune response [15, 16]. The more precise surface patch on an antigen that is directly contacted by the antibody is the epitope and the corresponding surface on the antibody in direct contact with the antigen is the paratope [17].

Some successful vaccines against virus infections have been based on attenuated but replicating variants of the pathogenic virus, for example, the smallpox vaccine and one form of polio vaccine. Attenuated variants of the simian immunodeficiency virus (SIV), which is closely related to HIV, have provided stronger protection than nonreplicating experimental vaccines against the wild-type virus, but the

mechanism of protection is not completely elucidated [18–20]. In other cases, recombinant proteins representing subunits of hepatitis B virus (HBV) and human papilloma virus (HPV) induce strong protection [21, 22]. The HPV vaccine consists of virus-like particles that may have advantageous properties both antigenically and immunogenically: they may present native neutralization epitopes well and be seen by the innate immune system as pathogen-associated molecular patterns [23]. But subunit immunizations have failed to protect against HIV type 1 (HIV-1) [1, 7, 8, 14, 24]. Only in the RV144 clinical trial, which combined viral proteins expressed from a canarypox vector with recombinant subunit protein boosts, was some modest protection observed. But the vaccine had not induced NAbs [8, 25, 26]. Therefore, the hunt is on for other antibodies and immune responses that might explain the limited protection. Many different antiviral effects of antibodies have been described that do not qualify as neutralization [27, 28]. This brings us to some semantic clarifications.

2. The Definition of Virus Neutralization

Definitions are arbitrary and contain no deeper knowledge than the proposed use of the defined term [29]. Therefore, the only reason to adhere to a strict definition of neutralization is that it may favor clarity and allow useful distinctions in the field of antiviral research. Neutralization, as discussed here, is defined as the reduction in viral infectivity by the binding of antibodies to the surface of viral particles (virions), thereby blocking a step in the viral replication cycle that precedes virally encoded transcription or synthesis [30, 31]. Classically the term was applied only to antibodies and fragments of antibodies, Fab and F(ab′)2, but later it has naturally been extended to single-domain antigen-binding recombinant fragments and natural nanobodies [32, 33]. Likewise the definition can be expanded to cover similar activities by soluble forms of viral receptors [34, 35], naturally occurring defensins, or other molecules of the innate immune system [36, 37]; it can be extended to lectins, either derived from plants or soluble recombinant and hybrid forms of mannose C-type lectin receptors (MCLRs) [38]. With regard to small organic molecules, if they are said to be neutralizing, it should be clarified whether that is meant to imply that they act by binding to the surface of the virion [39–49]. This review, however, only discusses antibody-mediated neutralization.

What does the definition not include? An antibody might bind to a budding virion, thus acting late in the viral cycle, thereby blocking the release of newly formed virus from the surface of an infected cell. Examples of antibodies acting like this are those directed to the neuraminidase on the influenza viral surface [50]. The enzyme releases progeny virus by digesting the neuraminic-acid moiety of the receptor for the virus. We shall see later why such an antibody, although its antigen decorates the virion surface, does not interfere at the beginning of the replicative cycle and therefore, per definition, does not neutralize.

Another semantic point is that an antibody to a receptor for the virus on the cell surface may block viral infection, but does not neutralize according to the definition: it does not bind to the virions, each of which, diffusing around in the extracellular space, would be potentially as infectious as in the absence of the antibody; instead the target cells would be rendered nonsusceptible. Hence, for clarity, some other term, such as infection-blocking antibody, should be used in that special case.

The usefulness of a strict definition becomes obvious in vaccine research when we ask by which mechanism vaccine-induced antibodies protect from infection. Antibody-dependent cellular cytotoxicity (ADCC) is a well-established effect that requires effector cells and counteracts viral infection by killing the virus-producing cell [27, 28]. The antibodies involved may be neutralizing, when tested in the absence of effector cells (natural killer cells) but often are not. And some neutralizing antibodies are not of the right isotype to mediate this effect. Antibody-dependent cell-mediated viral inhibition (ADCVI) has a more complicated relationship to neutralization, which may concomitantly occur in the assay used for measuring the effect. But to the extent that the inhibition depends on effector cells, it is not neutralization. Again, antibody isotype will affect the component in ADCVI that involves Fc-Fc-receptor interactions. Furthermore, the epitope location on the influenza virus haemagglutinin determines whether an antibody is merely neutralizing or also capable of mediating Fc-receptor interactions and thereby ADCC and protection *in vivo* in a mouse model [51].

HIV-1 can be transmitted directly from cell to cell via a virally induced connection known as the virological synapse [52–55]. It is convenient, however, to call the inhibition of cell-to-cell transfer something other than neutralization, even when it is mediated by NAbs [56]. The reason is that non-NAbs, including antibodies that interfere with the formation of the synapse by binding to cellular structures and antibodies that counter virion formation or release, might block this mode of transfer. It is also noteworthy that the relative efficiency of neutralization and block of cell-to-cell transfer differs among NAbs [57]. Observance of these distinctions makes for clarity.

Normally, neutralization is measured in the absence of complement, and the definition can certainly be made more stringent by making that a criterion. But it may be more useful to allow complement-mediated enhancement of neutralization as a legitimate category [58]; regardless, it is valuable to quantify that effect [59, 60]. Again, if complement factors reduce viral infectivity by binding directly to the viral surface rather than to antibodies already in complex with virions [61], this could also qualify within the wider definition of neutralization.

One consideration of biological and medical importance is that neutralization does not have to equate only what is measured in the neutralization assays *in vitro*. Although neutralization must be measured *in vitro*, we can fruitfully discuss how it operates *in vivo*. To infer that it is responsible for protection *in vivo* is more complicated and requires the use of antibodies without other effector functions or experimental models in which cell-dependent mechanisms are knocked out by other means [62–64]. And effects late

in the viral cycle may be harder to prevent in experiments *in vivo*: as always, the hypotheses can only pass ever more stringent tests but never be proven true. Semantically, though, there should be no barriers to discussing neutralization that occurs both *in vitro* and *in vivo*.

The part of the replicative cycle delimited by the definition encompasses many different steps, some shared by all viruses, others specific for certain groups. To infect a cell, a virion must attach itself to the cell surface. This can occur via ancillary attachment factors or directly via the major receptor used by the virus for entry; some viruses then interact with a second receptor, or coreceptor [65–79]. Naked viruses need to penetrate a cellular membrane to enter the cytoplasm or instead they may inject their genome through a membrane [80]; enveloped viruses must fuse their envelope with a cellular membrane in order to translocate their core and genome into the cytoplasm [79, 81–85]. Some viruses enter the cytoplasm directly from the cell surface; some depend on endocytosis for productive entry, sometimes because the reduced pH in the endosome triggers penetration or fusion [86]. If the virus enters directly from the cell surface, the genome-containing particle must again penetrate a barrier, the cortical cytoskeleton, which is an actin mesh with masks sometimes smaller than naked virions or viral cores [70, 87]. By entering after endocytosis, the virus surmounts this obstacle, delivering the capsid to a more central location. After these steps, the core may need to be uncoated, or to migrate to specific locations in the cell, before transcriptional or translational synthetic events take place, beyond which any inhibitory effects per definition no longer constitute neutralization.

3. Mechanisms of Neutralization

Neutralization has many mechanistic aspects: how the NAbs bind, whether they induce conformational changes, whether they irreversibly inactivate the viral proteins that mediate entry, and whether they are most effective against virions in suspension or after virion attachment to cells. Here, however, the mechanism of neutralization refers specifically to which early step in the viral replicative cycle is blocked (Figure 1).

If the NAbs prevent the virions from attaching to the targets cells, that is the mechanism of neutralization. If the NAbs block necessary receptor interactions after attachment, that is also a mechanism of neutralization; so is interference with any other obligatory step in the entry of individual viruses, such as coreceptor engagement, endocytosis, fusion, or penetration. One way to explore postattachment neutralization (PAN) is to let virus adsorb to cells at a low temperature that does not permit fusion or internalization and then to add the NAb. It should not be inferred, however, that PAN does not interfere with receptor interactions: some receptor contacts may first be established at the lower temperature, and then the NAb binds and prevents further necessary receptor recruitments during the warm-up [88, 89]. PAN might even reverse attachment. Still, investigating the capacity for PAN may contribute to the characterization of a NAb. That a NAb is capable of PAN does not, however, demonstrate PAN as the overriding mechanism at more physiological temperatures, nor does the lack of PAN at low temperatures exclude that it would occur at higher ones: receptor-induced epitope exposure on the entry-mediating viral proteins may require temperatures that do not block entry. If, nevertheless, PAN is detectable but less potent than preattachment neutralization there can be several explanations. First, it is conceivable that when receptor contact is already made, a higher occupancy by NAb on the remaining receptor-binding proteins for stopping

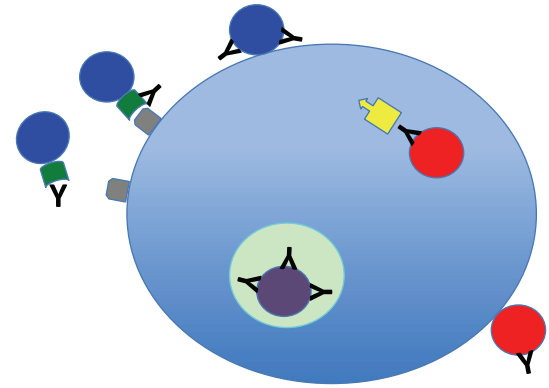


Figure 1: The mechanism of neutralization. Neutralization of enveloped viruses blocks viral attachment and entry. No other mechanisms are yet known; but entry can be blocked at different stages. The three blue virions to the right represent enveloped virus particles. The first has an IgG bound to its receptor-binding protein (green, for simplicity shown as a single copy). The bound NAb blocks the docking onto the receptor (grey) on the cell surface. The second virion has already established contact between its receptor-binding protein and the cell-surface receptor. The NAb binds to an epitope on the envelope glycoprotein (viral proteins with this function and topology are usually glycosylated) that may have become exposed after the receptor binding and blocks subsequent steps; these could be interactions with a second receptor or the fusogenic refolding of the envelope glycoprotein. The third blue virion is about to fuse with the cell membrane, but NAbs bound to membrane proximal epitopes on fusogenic proteins (not shown) prevent the completion of this process. The latter two interferences with entry could also occur in endosomes, but hardly the first, unless there are alternative attachment proteins the virus can bind to and thereby get internalized. The purple virion in the endosome is prevented by NAbs from fusing its envelope with the vesicular membrane. Alternatively, this purple virion could represent a naked virus particle, the penetration of which is prevented by the NAbs. The block of infection in the endosome could properly be called a postinternalization block of entry; for clarity, entry should refer to transfer of the viral core or capsid, or possibly only genome, into the cytoplasm. The red virion on the cell surface depicts a naked virion that binds to a cell surface receptor and injects the genome into the cytoplasm. This process may occur in vesicles or semisealed invaginations of the cell surface. If the NAbs have not prevented receptor interactions, they may interfere with the extrusion of the genome. The red virion in the cytoplasm has penetrated an endosomal membrane in complex with the NAb, allowing binding to TRIM21 (yellow box with arrow), which mediates the ubiquitination of the complex, targeting it for proteasomal degradation. This fairly recently discovered effect constitutes the clearest example so far of a postentry mechanism of neutralization.

entry is required than on the free virion. Second, kinetics come into play: the on-rate for the NAb binding will need to be higher when the attachment-entry process has begun than when the NAb binds in the absence of cells, and hence a higher NAb concentration is required if the on-rate constant is the same. Third, PAN may act partly by competition with receptors, thereby reversing attachment, which may require higher concentrations of NAbs than preventing it, because of the valency of the virus-receptor interactions and the strengthening of initial attachment by subsequent receptor recruitment.

Many of these mechanisms are experimentally confirmed for various viruses and for infection of target cells under different conditions. For example, NAbs have been demonstrated to block attachment of rhinovirus to HeLa cells [90]. The situation with poliovirus appears more complicated. But more recent elucidation of the mechanisms of picornaviral entry may shed light on precisely which necessary steps are prevented by neutralization and whether neutralizing mechanisms differ within the viral family [39–41, 65, 91, 92]. In the studies of polivirus neutralization, some NAbs were found not to block attachment, but to block endocytosis partially, to induce a pI shift in the virion, or to prevent transcription [58, 93–96]. One study found that among NAbs inactivating unattached virus, only the bivalently binding minority were capable of PAN; the others were rendered capable of PAN through cross-linking of their Fc portions. Furthermore, neutralization coincided with the prevention of a structural change in the 135S RNA-containing virion and of the genomic extrusion that normally produces 80S empty capsids in the uninhibited infection process [97]. Finally, the capacity to aggregate poliovirus particles was recorded as an exception among the NAbs [97].

Aggregation of virions by antibody has been regarded as an effect to distinguish from neutralization [58]. But in so far as it reduces infectivity it would qualify as a limited neutralizing effect, albeit with a complex dependence on antibody and virion concentrations. Typically, virion aggregation, as a function of antibody concentration, describes a dome-shaped curve: at low concentrations of antibodies, cross-linking of antigens on individual virions is favored, at higher concentrations virions are bridged, but when the epitopes on the virions come close to being saturated, cross-linking can no longer occur. A quantal assay may be required for measuring the loss in infectivity: a large aggregate would diffuse more slowly than virions but might sediment onto susceptible cells; fewer cells may end up being infected than by a monodisperse suspension of virions, but those that do may attain a higher multiplicity of infection. In a quantal, that is, focus-counting, infectivity assay this could give a distinct reduction of infectivity [98]; in assays based on the production of viral antigens or activation of reporter genes the effect could be smaller or absent. Still, a reduced infectivity would count as neutralization because it stems from how the virus is prevented from reaching and entering its target cells. *In vivo*, aggregation might have differential effects depending on target cell availability and requirements for diffusion, but their phagocytosis of aggregates, an ancillary inhibition not attributable to neutralization itself, might enhance the antibody-mediated inhibition.

The situation is different when antibodies do not aggregate the virions but block attachment and entry by competing with receptor interactions of the virus or otherwise counteract the function of the viral proteins that mediate fusion or penetration: there would be no basis for a dome-shaped inhibition curve. Then we might instead predict that the higher the occupancy such antibodies reach on the surface of the virions the less likely the viral infection will be; alternatively, there is a definite threshold of occupancy above which the infectivity is completely eliminated. These considerations are formulated within *the occupancy theory of neutralization*, which has plausible links to the blocking of different attachment and entry steps as neutralizing mechanisms [31, 99, 100].

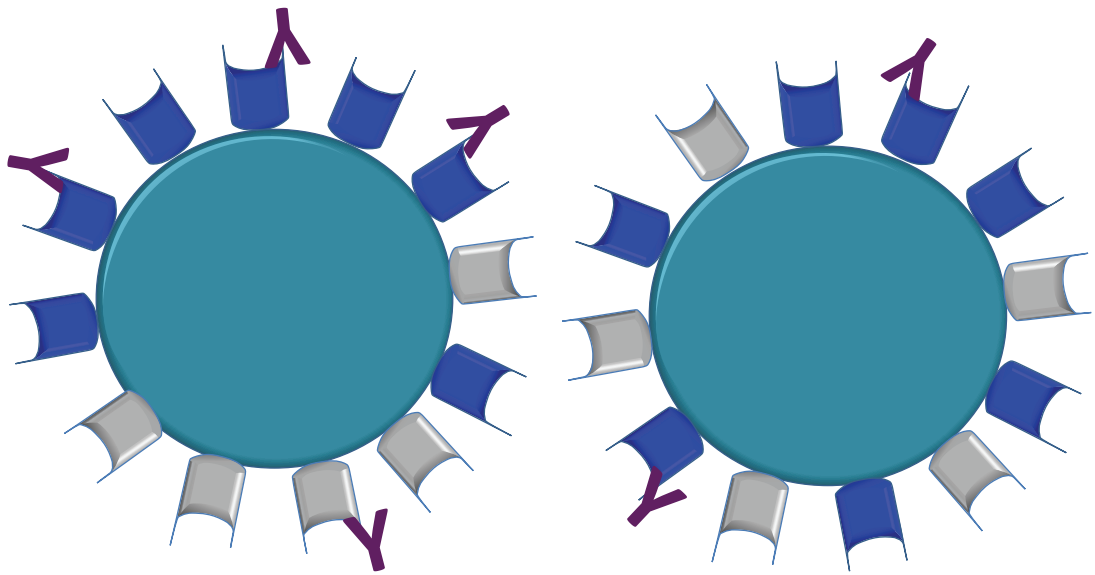
4. The Occupancy Theory of Neutralization

There are two kinds of scientific hypotheses: some are testable; others are detestable. Occupancy theories of neutralization come in different versions with varying potential for direct experimental testing. Strong versions suggest that IgG molecules because of their bulk impede the function of viral attachment- or entry-mediating proteins. One antibody molecule per viral protein subunit or even per oligomer may be sufficient. If a certain number of unoccupied such viral proteins were required for infection, then all virions with higher occupancies would be neutralized. The minimum neutralizing occupancy would constitute the neutralization threshold. So far so good, but there are several complications.

The binding to defective entry-mediating proteins on the virions would not be directly relevant to neutralization (Figure 2). Antibodies that can only bind to nonfunctional forms of entry-mediating proteins may never be neutralizing by themselves [101–104], although they might potentiate NAbs by making the sterically blocking coat on the virion thicker. But some NAbs can bind both to functional and defective targets, and their added capacity to do the latter may not be irrelevant to neutralization: it may increase their avidity and thereby indirectly enhance the occupancy on the functional targets.

As outlined in Figure 2, the effects of binding to functional entry-mediating oligomers are also complex. The unoccupied functional sites might need to be clustered in one area of the virion surface in order to function. But they may be moveable so that a sufficient number can be recruited into an entry complex together with receptors after the initial docking of the virion onto a single or too few receptor molecules [31, 84, 105–113]. If these viral proteins are not moveable, however, and they are unevenly distributed over the virion surface, it may only be when NAbs bind within the clusters that they have a neutralizing effect. All of these complications would be expected to soften the apparent thresholds of neutralization, even if it is postulated that a virion is either completely neutralized or not [84, 105, 108–110].

A different view would be that any antibody occupancy would dent the propensity to infect and the fewer the



(a) (b)

Figure 2: Neutralizing occupancies over heterogeneous populations of enveloped viruses. Two enveloped virions are pictured. Each has twelve glycoprotein spikes schematically displayed, for clarity only at the circumference. Functional spikes are shown in blue, decayed or otherwise nonfunctional ones in grey. Both virions have seven functional and five nonfunctional spikes but with different distributions over the two virion surfaces. If a virion requires a certain number of spikes in contiguity to form an entry complex and the spikes cannot move freely over the virion surface, the two different distributions will confer different neutralization sensitivities. The virion to the left is neutralized: three NAb molecules inactivate the constellation of active spikes and one binds redundantly to an inactive spike. The virion to the right is also neutralized but by only two NAb molecules, one inactivating a group of three spikes (three adjacent ones being postulated here to be the bare minimum for entry) and one binding redundantly to a spike that is functional but inert through lack of active neighbors. Effects of this sort could blur critical occupancy thresholds and reduce the steepness of neutralization curves in experiments with phenotypically mixed virus, of which the virions carry random assortments of antigenic and nonantigenic subunits of the envelope glycoprotein oligomers. Heterogeneity of the number (not shown) and distribution (shown) of functional entry-mediating viral proteins may explain how different occupancies are required for blocking viral entry. Some of these considerations apply also to naked viruses.

unoccupied functional entry-mediating molecules, the lower the infectivity. Such a proportional relationship, applied to the full range of occupancies, we can call the *incremental* model, as opposed to models that postulate a threshold, which we can name *liminal* [105, 114]. Now, both models can be formulated mathematically in their pure form, but fitting them to data suggests that mixed models are the most realistic [31, 84, 105–110]. Thus, there may be an absolute minimum of a close constellation of unoccupied entry-mediating molecules. But when the constellation is larger than necessary, any antibody binding still dents infectivity. And if the redundancy of entry molecules is huge, that denting may be negligible in relative terms. In other words, there is neither a strict proportionality, nor a sharp threshold.

One version of the occupancy theory suggests that neutralization will occur when NAbs achieve a sufficiently dense coat on the virions [99]. This coating theory has several distinct implications. It invokes steric hindrance of access not only to the occupied entry-mediating molecule but to adjacent ones as well. It implies a linear relationship between virion surface area and the minimal number of NAb molecules required for neutralization. This linearity holds up approximately [99]. It would explain why antibodies to influenza virus neuraminidase do not neutralize [50, 115]: the density of that antigen is too low; there are four times as many hemagglutinin as neuraminidase molecules on the virus surface [99]. Some degree of neutralization does, however, result from cross-linking the antibodies bound to neuraminidase by anti-Fc antibodies [116]. Maybe the two-layered antibody coat gives greater steric interference.

Likewise, rabies virus is not neutralized by an antibody that recognizes a minority conformation of its surface glycoprotein. But when the majority is converted to the antigenic conformation by reduced pH or elevated temperature, the virus is neutralized by that antibody [117]. This is strong support for the coating version of the occupancy theory. Analogously, the theory would explain why antibodies to some cellular passenger antigens such as ICAM-1 and MHC on the surface of HIV or SIV virions do neutralize, albeit in a largely complement-dependent manner [118–120]: the antigen is so abundant that its coating partly impedes access to the few envelope glycoprotein trimers.

If steric interference can occur intermolecularly, the blocking of a receptor-binding site by the binding of an IgG molecule elsewhere on the same entry-mediating viral protein is even more plausible. NAbs that do not bind to the main receptor-binding site on HIV-1 Env appear to block receptor interactions: if the target cells lack ancillary attachment

factors, antibody binding anywhere on the receptor-binding subunit of Env suffices to block viral attachment to cells, that is, when attachment is mediated by the primary receptor. Thus, under those conditions, steric or direct hindrance can prevent the first step in replication [121]. Still it matters on which subunit the epitope is located. Some NAbs are directed to epitopes close to the viral membrane in the transmembrane protein. They do not interfere with attachment to cells that lack ancillary attachment factors, and hence apparently not with receptor binding [121]. In conclusion, binding close to the receptor-binding site may be required for an indirect block of receptor interactions.

The theory also implies that the potency of NAbs closely correlates with their affinity for the native oligomeric form of Env on the viral surface, and this too largely holds up for HIV-1 [100, 103]. Again, an exception would be NAbs directed to the membrane proximal region in the transmembrane protein [122, 123]. Those epitopes may only become exposed or fully antigenic after receptor interactions have induced some conformational changes in Env. Analogously, the potency of peptide inhibitors binding to the transmembrane protein does not correlate with affinity for the protein but with the on-rate constant [124]. In summary, affinity of NAbs for native entry-mediating molecules correlates well overall with neutralization potency, but in some cases affinity for a receptor-induced transient form of the viral protein determines neutralization.

The coating theory furthermore implies that, although the binding of NAbs may induce conformational changes, such effects should not be necessary for their neutralizing capacity. This prediction clashed with a long tradition of research, particularly on picornavirus [58, 94–96]. New data did indeed indicate a lack of correlation between neutralization and the capacity to induce conformational changes in rhinovirus [125], which would suggest that the conformational changes are epiphenomena, accompanying the binding of some NAbs but not others. A potent NAb against HIV-1, directed to an epitope separate from the CD4-binding site, appears to block CD4 interactions allosterically, as detected with soluble forms of the Env trimer [126]; whether such conformational changes are necessary also at the level of virions coated with the NAb would be harder to investigate. Perhaps allosteric interference with receptor interactions can add inhibitory power to mere steric hindrance.

Yet another implication of the occupancy theory in general and its coating version in particular is that non-NAbs should not be able to block NAbs. Although studies on Japanese encephalitis virus suggested such interference [127], non-NAbs directed to the HIV-1 Env protein have been shown not to block neutralization by NAbs even when they are directed to overlapping epitopes. What are the explanations? The occupancy theory implies that all NAbs that bind to functional entry-mediating viral proteins should neutralize. Indeed, the non-NAbs that bind to epitopes overlapping known neutralization epitopes on the HIV-1 Env subunit gp120 have been shown not to bind to gp120 in its trimeric native context [102, 103].

For influenza hemagglutinin and poliovirus capsid proteins, overlapping epitopes of NAbs and non-NAbs have also been described, but the crucial question is whether the non-NAbs really bind to the native protein on the surface of infectious virions [93, 128]. Intriguingly, it has also been observed that a mannose-binding lectin can block neutralization by a NAb directed to a mannose epitope on HIV-1 Env [129]. If the blocking of neutralization by non-NAbs ever occurs, an explanation might have to be sought in conformational changes that the NAb confers and the non-NAb does not and *vice versa*. The focus would shift back to whether particular conformational changes in the antigen are instrumental to neutralization. Thus the non-NAb would protect the antigen until it can be competed off by receptors. But why would it not be competed off by the NAb?

A very recent study indicates how neutralization-blocking non-NAbs might act, although it reports effects in the greyer area of shifts in potency and efficacy. Certain mutations in HIV-1 Env reduce the degree of neutralization by a broadly active and potent NAb (10E8 [122]) directed to a membrane-proximal epitope in the transmembrane protein. Although these mutations thus do not convert the NAb to a non-NAb, it is possible to study how the binding to the mutant virions affects their sensitivity to other NAbs, that is, how the residually infectious virus in complex with this still partly active NAb is neutralized [123]. The NAb enhances the neutralization by some antibodies to other epitopes in gp41 but reduces that by others, including those directed to adjacent epitopes as well as those specific for the CD4-binding site. The stoichiometry of the binding of the NAb to mutant trimers is lower than for wild-type Env: two instead of three paratopes bind [123]. This low degree of binding stabilizes the trimer and may have distant allosteric effects such that the binding of other NAbs is facilitated or impeded. Hence, this intriguing case may thus uniquely show that weakly neutralizing antibodies can counteract rather than add to the action of stronger ones. If the allosteric mechanism involves reduced affinity for the distant affected epitopes, it rather corroborates the occupancy theory than undermines it.

Also pertinent to the occupancy theory is which Abs can capture virions. Both NAbs and non-NAbs can capture HIV-1 virions, but NAbs preferentially capture infectious virions [130]. Paradoxically, though, non-NAbs, which do not block neutralization, block capture [131]. That raises the question why the binding of NAbs to functional trimers alone does not mediate the capture of the virions. Perhaps some NAb binding to gp120 makes it dissociate from the transmembrane protein gp41 that anchors it in the viral membrane [132, 133]. Again, if all NAb binding had that effect, it would be hard to explain the preferential capture of infectious virions by NAbs.

As mentioned, in the case of HIV-1, some neutralization epitopes reside in the transmembrane protein, but other epitopes there are occluded by gp120; and although the latter are immunogenic, the antibodies directed to them do not neutralize because they can only bind to nonfunctional stumps of trimers after the shedding of gp120 [103, 134, 135]. This all agrees with the basic occupancy tenets. Even the enhanced binding of NAbs during transient exposure of the epitopes close to the membrane agrees with the theory. Although these epitopes are present on functional trimers,

they are only weakly antigenic in the native form of the Env spike.

Several NAbs to enveloped viruses block late steps in the entry process, that is, the fusion of the viral with the cellular membrane. And this block does not have to occur at the cell surface. If the antibody binding allows receptor interactions to some extent, the virus may get endocytosed and the fusion that is in some cases triggered by the lowering of the pH in the endosome is delayed, and then the virus is shunted towards lysosomal destruction before it has fused and extruded its core and genome into the cytoplasm. This scenario has been exemplified for West Nile virus [136].

HIV-1 may also depend on internalization for complete fusion [83–85, 137] and hence could be subject to neutralization by antibodies that permit endocytosis of the virion but interfere with late fusion steps. We could call this intracellular neutralization, with the important distinction that it is still entry—fusion preceding translocation of the core into the cytoplasm—that is blocked. But the definition of neutralization, as outlined initially, would allow for somewhat later steps to be blocked, namely, at the early postentry stage, in the cytoplasm, before the transcription of the viral genome or translation of viral products. Do such mechanisms ever occur?

5. The Naked Truth about Postentry Neutralization

Postentry mechanisms of neutralization have been asserted repeatedly about picornavirus and influenza virus [138, 139]. But definitive evidence was lacking and many a virologist may have regarded neutralization as in practice synonymous with antibody-mediated inhibition of attachment and entry. Then a new intracytoplasmic mechanism of neutralization was discovered [140–142]. Studying adenovirus, James and colleagues found that its neutralization is greatly dependent on the presence of tripartite motif-containing protein 21 (TRIM21) in the target cells. TRIM21 is located in the cytoplasm, has a strong affinity for IgG, and ubiquitinates the antibody-antigen complexes that it captures, targeting them for destruction by the proteasome [141, 142].

One surprising aspect of how the intracytoplasmic neutralization works is that the adenovirus particle as an intact complex with the NAb must translocate across a vesicular membrane into the cytoplasmic compartment. At least one important route of adenoviral entry is internalization through macropinocytosis followed by penetration of the vesicular membrane. Hence as long as the macropinocytic vesicle remains intact, and the capsid ligated by the NAb has not penetrated from there, TRIM21 does not gain access to the complex. Only once penetration occurs can NAb-TRIM21 contact be established. Quite conceivably a single NAb molecule might be sufficient for targeting the complex to TRIM21, but it also seems possible that targeting would be enhanced by a greater number of NAbs bound [143]. Those considerations also raise questions of how many NAb molecules can traverse the membrane together with the virus particle; one hypothesis would be that a high occupancy of NAbs prevents entry, by blocking attachment, receptor interactions, or a later penetration step, but that TRIM21 acts as a safety net, enabling the neutralization of virions with low NAb occupancy. That would, however, imply substantial TRIM21-independent neutralization at the highest NAb concentrations, which does not seem to happen [143]. These new problems will be further explored in the analyses of stoichiometry and efficacy of neutralization below.

How general could this mechanism be? It would seem to be strictly limited to naked viruses. For when enveloped viruses fuse, NAbs bound to the viral surface proteins do not gain access to the cytoplasm. And although capsid proteins of enveloped viruses elicit strong antibody responses during infection and when expressed from vaccine vectors (see, e.g., [144–146]), these antibodies cannot bind to their antigens when the virion is intact. They would have to translocate into the cytoplasm on their own. Indeed, the discovery of the TRIM21 mechanism seems to create a new dichotomy of virus neutralization, a mechanistic divide between potential NAb effects on naked and enveloped viruses. But then the TRIM21 mechanism may not apply to all naked viruses either. If picornavirus injects its genome into the cytoplasm, and the capsid therefore never enters [80, 97], the result would be an absence of antibody-capsid complexes in the cytoplasm and hence a lack of targets for TRIM21.

Some naked viruses and the capsids of enveloped ones need to be uncoated after entry for replication to proceed. It is therefore not farfetched to imagine that the virus could have taken advantage of ubiquitination by TRIM21 and other factors to facilitate this step. If degradation of the naked virion in complex with the NAb is too slow to prevent escape of the genome towards the next replicative step, or if the capsid of an enveloped virus gets ubiquitinated through an alternative interaction, the virus might benefit from the cellular assistance in its uncoating. But the core of HIV-1 is degraded by the proteasome to a large extent in uninhibited infection: the degradation causes a net loss in infectivity [147]. And at least with adenovirus, the neutralizing effect seems to dominate over any potential advantage to the virus.

TRIM21-dependent neutralization also has implications for whether some antibodies can block neutralization. If IgA and IgM, specific for neutralization epitopes overlapping those of IgG NAbs, were incapable of ligating TRIM21, they would conceivably be able to block TRIM21-dependent neutralization by IgG, provided they were of high enough affinity and present at sufficient concentrations. But evidence suggests these Ab classes, just like IgG, can interact with TRIM21 [140, 142]. That capability would explain their inability to block this mechanism of neutralization, a very different explanation from that of why antibodies rarely block the neutralization of enveloped viruses.

Could no analogous mechanism operate against enveloped virus? One effect that might come closest is the binding of NAb-virion complexes to Fc receptors, followed by endocytosis, and ultimately lysosomal degradation of the virus. This antibody-dependent routing of virus would qualify as neutralization according to the definition. With HIV-1, for example, internalization depending on low NAb occupancy and subsequent loss of infectivity were observed, albeit not categorized as neutralization, perhaps because the

effect was too weak compared with regular neutralization [148]. But it should be noted that this mechanism would prevent entry: viable genome-containing cores would not enter the cytoplasm. The routing to lysosomal degradation may be inefficient because it is outcompeted by productive entry, which can occur across the endosomal membrane. Indeed, that may be the regular site of productive entry for HIV-1 [84, 85, 137]. Conceivably, the antibody in complex with the Fc receptor would block the fusogenic Env-receptor interactions by steric hindrance and thus delay fusion until degradation in the lysosomal compartment starts. Still, it might require lower occupancies by antibody molecules on the virions than the regular entry block, just as the much more vigorous TRIM21 mechanism would. Enveloped viruses among themselves also provide contrasting examples: low occupancies on flaviviruses can mediate enhancement of infectivity, whereas high occupancies by the same antibodies cause neutralization [81, 149–155]. We shall return to these considerations when discussing stoichiometry more comprehensively below.

The TRIM21-dependent postentry mechanism of neutralization and the occupancy-limited, entry-blocking mechanisms have quite distinct implications for classical and newly studied aspects of neutralization and will need to be contrasted continually. As an illustration, the occupancy theory might explain lack of neutralization by antibodies even though they bind to infectious virions: they might achieve too low occupancies or bind only to sites that are not functional in entry. In contrast, the TRIM21-dependent mechanism would not explain such lack of neutralization as long as TRIM21 recognizes the Fc portion of the antibody. For ubiquitination would not seem to require threshold levels of antibody occupancy, although quite plausibly the more the antibody bound, the greater and faster would be the degradation of the capsid. Nor would TRIM21 distinguish between antibody bound to functional entry-mediating molecules and other antigens. Perhaps some antibodies bound to capsids fail to get translocated into the cytoplasm; they would dissociate and let the unbound capsid enter, or else the antibody would neutralize at an earlier step. Clearly, the TRIM21 breakthrough not only explains much but also raises intriguing new questions.

In the context of the novel mechanism, it is a provocative observation that genetic antibody deficiencies seem to predispose for greater vulnerability to infection by naked viruses than by enveloped ones [142]. Is postentry inhibition prevalent among naked viruses? Does it provide a safety net or constitute the major defence line? Or are enveloped viruses more vulnerable to cellular immunity in addition to the antibody responses?

Those questions will have to be left unanswered here, but the TRIM21-dependent mechanism has intriguing connections to the quantitative aspect of neutralization to which we do not turn.

6. Kinetics of Neutralization

The reason for this section is twofold: first, to try to undo the damage of erroneous inferences from the kinetics of the neutralization reaction itself; and second, to clarify how this aspect is distinct from or related to other, less trivial, kinetic aspects of neutralization.

To view the neutralization of virions in suspension by antibody in solution as a chemical reaction requires some questionable assumptions. Thus, it must be assumed that at some point the binding events between the reactants convert the virions from infectious to noninfectious, an effect as black and white as the formation or breakage of a covalent bond. As already alluded to, virions (although here virus species are likely to differ substantially) may have a spectrum of propensities to infect, and neutralization may be a shift in such propensities, that is, not an all-or-nothing effect. But if we accept the premise that neutralization is a complete loss of infectivity of the individual virion, we could seek to know the *molecularity* of the neutralization reaction, that is, how many antibody molecules must bind to achieve neutralization. Molecularity is related to, but cannot be inferred from, the order of the reaction, which is a kinetic concept. The order can be empirically determined if the concentration of the free reactants are monitored together with the rates. Thus, the rate of the neutralization reaction, r, would be

$$r = k\,[\text{virus}] * [Ab]^n, \tag{1}$$

where k is the rate constant, [virus] and $[Ab]$ are the concentrations of the free reactants (which hence both decrease), and n is the order of the reaction in antibody concentration, which does not have to be an integer. Since virions are already assumed to act alone, the reaction is first order in virion concentration (neutralization through aggregation would be complex in this scheme). What remains to be determined is n. In attempts to infer the molecularity of the reaction, the rate of neutralization over time has been monitored as a function of antibody concentration. Values of n close to 1 have been observed and the conclusion has been drawn that a single antibody molecule inactivates one virion [156–158]. The fallacy is that the free antibody concentration is not recorded and its changes could not be measured because of the vast molar excess of antibody over virus. It is a classic situation of pseudo-first-order kinetics. Since [Ab] does not change significantly, the following approximation is true:

$$r \approx k'\,[\text{virus}]\,, \tag{2}$$

where $k' \approx k\,[Ab]$ is the pseudo-first-order rate constant for virion concentration. The data say nothing about the order in antibody concentration. A second flaw is the assumption that epitopes can be divided into critical and noncritical but that somehow only the binding to the critical epitopes would be reflected in the neutralization kinetics. This does not make sense: if binding is random and of equal affinity to the two kinds of epitopes, a certain number of noncritical epitopes would be bound with the same occupancy as for the critical epitopes. Hence, the order was erroneously obtained from kinetics and so-called single-hit molecularity was mistakenly inferred from the order; to cover these unjustified leaps the term *single-hit kinetics* is sometimes used. It should be noted that the original paper studied one naked and one enveloped virus and observed similar kinetics for these. The enveloped virus was western equine encephalitis virus [159].

The naked virus was poliovirus. Could these old suggestions have anything to do with the new discovery of the TRIM21 effect, which might potentially apply to several naked viruses?

No, that would be a specious convergence. TRIM21 might not contribute to poliovirus neutralization anyway, although the virus is naked, if its genome is extruded by transmembrane injection rather than translocation of the capsid together with any bound NAb into the cytoplasm. Hence, if it should turn out that TRIM21 allows single antibodies to mediate neutralization of some viruses, that outcome would be an important advance in knowledge. But it would shed no light on any surprising putative single-hit phenomena based on the kinetics of neutralization because there were no such phenomena, only flawed interpretations.

An earlier paper correctly described the consequence of the vast molar excess of antibody over virions (and over epitopes): as the virion concentration is varied over a wide range, the proportion that is neutralized by a fixed antibody concentration remains constant. This relationship was called The Percentage Law [160]. Those observations illustrate the basis for pseudo-first-order kinetics: the proportion of antibody lost by binding is negligible.

Another approach was taken to explore how many antibody molecules must bind before neutralization occurs. Thus on a curve for neutralization over time, the first segment of the curve was scrutinized for signs of any shoulder. If a shoulder was observed, it was taken to suggest that more than one antibody had to bind before infectivity was abolished for any virion. Sometimes such a shoulder was observed, sometimes not [161]. The difficulty lies in obtaining the requisite precision of data for a sufficiently early part of the curve. And even with the most precise such data, interpretations are not incontrovertible. The very method of stopping the neutralization reaction, namely, rapid dilution, is unsatisfactory since it makes the results contingent upon the degree of irreversibility. And if the NAb is used at a concentration below its K_d, there might be a shoulder even for very low occupancies, because the rate of binding would be so low. Furthermore, a single NAb bound to the smallest viruses would constitute a higher occupancy than several NAbs bound to larger viruses. Thus single- and multihit thresholds could look the same.

A different possible cause of a shoulder is that an antibody needs to induce changes in the viral antigen that are slower than binding; a lack of a shoulder could mean that virions lose some propensity to infect with the first binding events but are not completely neutralized as the single-hit hypothesis would suggest; the data would not distinguish between a partial dent in the infectivity of many from a complete loss for a few. The reasoning here is analogous to the distinction between incremental and liminal models. Only a high and homogeneous threshold might show up as a broad shoulder on the kinetic curve.

The mistaken single-hit interpretations led to the search for mechanisms such as inactivating signals from bound NAbs to the interior of the virion for enveloped viruses and conformational shifts in the whole capsid for naked viruses. Generally, the hunt was on for postentry mechanisms. For a block of receptor interactions and entry seemed less readily explicable by single-hit molecularities, at least in the case of enveloped viruses.

As a practical consequence of the single-hit hypotheses, the neutralizing occupancies will be low, except if the virus is also postulated to have only a single relevant antigen molecule [84, 105, 106, 111]. And the lower the occupancy required, the easier would be the task of inducing protective binding titers of NAbs by vaccination: titers and occupancies are the products of antibody affinities and concentrations. The misinterpretations can misguide vaccine research.

7. Kinetics of Binding: The Example of HIV-1 Env

The kinetics of the binding of NAbs and other antibodies to surface proteins of viruses are more readily studied than the kinetics of neutralization. One technique that allows kinetic measurements is surface plasmon resonance (SPR) [162–167]. With this technique, the antigen or the antibody can be immobilized to a sensor chip. If the antigen is immobilized, the antibody in solution is injected to flow over it. Binding produces a change in the angle of the reflection of polarized light, which is monitored and translated into a resonance signal, proportional to the mass of protein that has bound. This technique has been used for measuring antibody binding to the neutralization targets of various viruses, including the HIV-1 Env glycoproteins. Modeling of the binding at different concentrations gives the on-rate constant, k_{on}, the off-rate constant, k_{off}, and their ratio, $k_{\text{off}}/k_{\text{on}} = K_d$, the dissociation constant, a reciprocal measure of affinity; furthermore, since the maximum equilibrium binding is approached and can be extrapolated, the stoichiometry of binding can also be estimated. Such studies on the binding to conformationally flexible viral envelope glycoprotein oligomers that mediate entry into susceptible cells have recently become more relevant to neutralization through improvements in the mimicry of native antigens.

The antigenicity of the receptor-binding subunit, gp120, of the HIV-1 Env trimer has been studied extensively, but, as mentioned, many of the epitopes that gp120 exposes are shielded on the native trimer [168]. Likewise, the uncleaved precursor of Env, although it trimerizes, differs antigenically from native functional trimers [102]. Still, in order to produce soluble trimers, truncated N-terminally of the transmembrane segment, that do not disassemble, a common approach is to delete the cleavage site between the subunits and to add extra trimerization motifs C-terminally of the truncation [169–174]. But these uncleaved soluble trimers do not adopt native-like structures and are therefore poor antigenic mimics of functional spikes.

As an alternative approach, proteolytic processing has instead been enhanced by modifying the cleavage site and by coexpression with the protease furin; but to maintain the integrity of the trimer of heterodimers, a disulfide bond has been added to link gp120 covalently to the truncated transmembrane protein, gp41 [103, 126, 175–180]. These soluble trimers structurally mimic native trimers on the surface of the virion, as assessed by electron microscopy

[102]; furthermore, their three-dimensional structure has been determined to near-atomic scale resolution, in complex with Fabs of different NAbs [181, 182].

For SPR studies, several dangers of artifactual results lurk in various approaches. Even with trimers that mimic the native spikes structurally, if they are immobilized directly to the SPR chips by covalent, such as amide, coupling their antigenicity will be perturbed. Hence, it is advantageous to add His or epitope tags C-terminally at the truncation so that the trimers can be captured by Ni^{2+} or antibody that is immobilized on the chip. When the cleaved and stabilized Env trimers are immobilized by such capture, they bind NAbs active against the corresponding strain of the virus excellently and non-NAbs negligibly. Take different antibodies directed to the CD4-binding site and to the variable V3 region as examples. Both groups contain antibodies that are neutralizing and others that are nonneutralizing against particular strains of HIV-1. They bind equally well to the monomeric Env subunit gp120 and to uncleavable mutant trimeric forms of Env derived from the same strains. But only the neutralizing ones bind well to the trimers derived from the strain they neutralize. Some NAbs to particular epitopes do not bind to monomeric or nonnative forms of Env: they are trimer-specific, whereas non-NAbs regardless of epitope fail to bind the native-like trimers (Figure 3) [102, 103, 175, 183]. Hence, what NAbs have in common is that they recognize native-like entry-mediating viral proteins; what non-NAbs have in common is that they do not. How they differ or resemble each other in the recognition of other forms of Env is then irrelevant. This supports the occupancy theory of neutralization [31, 99, 106, 184]. Possibly, some antibodies that have been observed not to neutralize other viruses, in spite of binding to virions, may also turn out to recognize only nonfunctional forms of the viral surface proteins.

Because of the richness of the information obtained by SPR, NAbs with similar affinity but widely different kinetics of binding can be identified. Such characterization of binding goes beyond mere occupancy and ushers in the possibility of testing more dynamic neutralization theories: how do the kinetics of NAb and receptor binding together mold the efficacy of neutralization?

When the binding of IgG and Fabs is compared by SPR, the specific models for bivalent or monovalent binding can be explored. We know little of the density of Env trimers on infectious HIV-1 virions, let alone the ratio of functional to defective or decayed trimers. That ratio may also change as the virions age. But the immobilization of Env on the SPR ship can be precisely controlled and translated into trimer densities that can be compared with and adjusted to the observed densities of Env on HIV-1 virions. With improved knowledge, simulations of trimer densities on virions, and thereby realistic average trimer distances, may render the measured degree of bivalency of binding to the antigen on the SPR chip relevant to neutralization. Some NAbs, however, can bind to both defective and native-like trimers, and this would enhance binding through bivalency; other NAbs recognize only the native-like trimers and that would limit their avidity.

Other viruses than HIV-1 tend to have higher ratios of half-maximal inhibitory concentrations of Fab over those of IgG [185]. The contribution of the bulk of the Fc portion, possibly through steric effects, can be ascertained by comparing Fab and $(Fab')2$ in neutralization. But the binding of IgG can also be strengthened by Fc-Fc interactions, and this could be evaluated by SPR [186, 187]. Likewise, Fabs and smaller single-chain constructs could be compared. Through these combined comparisons, the avidity and bulk effects would be distinguished. Such background knowledge can be compared with and corroborated by simulations of the trimer density on the virion surface.

It should be noted that the degree of bivalent binding, which enhances the potency of NAbs by reducing the off-rate of their binding, is favored by high densities of antigen on the virion surface [185]. Such an effect would counteract the relative neutralization resistance stemming from a high redundancy of functional entry-mediating molecules [106, 188–191]. But the counteracting selective forces are not symmetric: that some NAbs bind equally well to functional and nonfunctional entry-mediating molecules would increase bivalency when extra nonfunctional oligomers are present, but the redundancy effect requires functional oligomers. These factors may play out in the evolution of natural viral variants with varying degrees of neutralization sensitivity.

One SPR-based study of the simian immunodeficiency virus (SIV) and its Env protein gave several surprising results. NAbs and non-NAbs bound with similar kinetics, and therefore similar affinity, to recombinant, soluble Env protein [192]. But this was uncleaved Env protein, later found to expose nonneutralization epitopes; and the SPR was based on immobilized antibody with trivalent Env in solution, which does not simulate the potentially bivalent but often monovalent binding of IgG to virions. Also surprising at the time of publication was that Env from a neutralization resistant strain bound with faster on- and off-rates to NAbs (and non-NAbs) than did Env from a sensitive variant. The on- and off-rate differences canceled each other out, yielding no net affinity difference. This would point to a greater importance of low off-rates in conferring neutralization sensitivity than high on-rates, although the other caveats about the SPR conditions might invalidate comparisons with neutralization. Certainly, the relative influence of the on- and off-rate constants may vary. But how they vary will be determined by the length of the preincubation with NAb in the neutralization assay. That raises questions about which conditions are most relevant to protection *in vivo*.

8. Kinetics of Entry and Virion Decay: Mode of Neutralization

A classic neutralization assay comprises four stages. First, virus and antibody are incubated together. Second, the virus is allowed to adsorb to target cells. Third, viral replication proceeds to produce viral product or induce the expression of a reporter molecule. Fourth, the product is measured in an assay and converted to a signal and compared with no-antibody and no-virus (background, noise) controls [31]. The first two phases can be varied; the first one can even be eliminated. The second one can be performed at reduced temperature, so that internalization and entry are prevented.

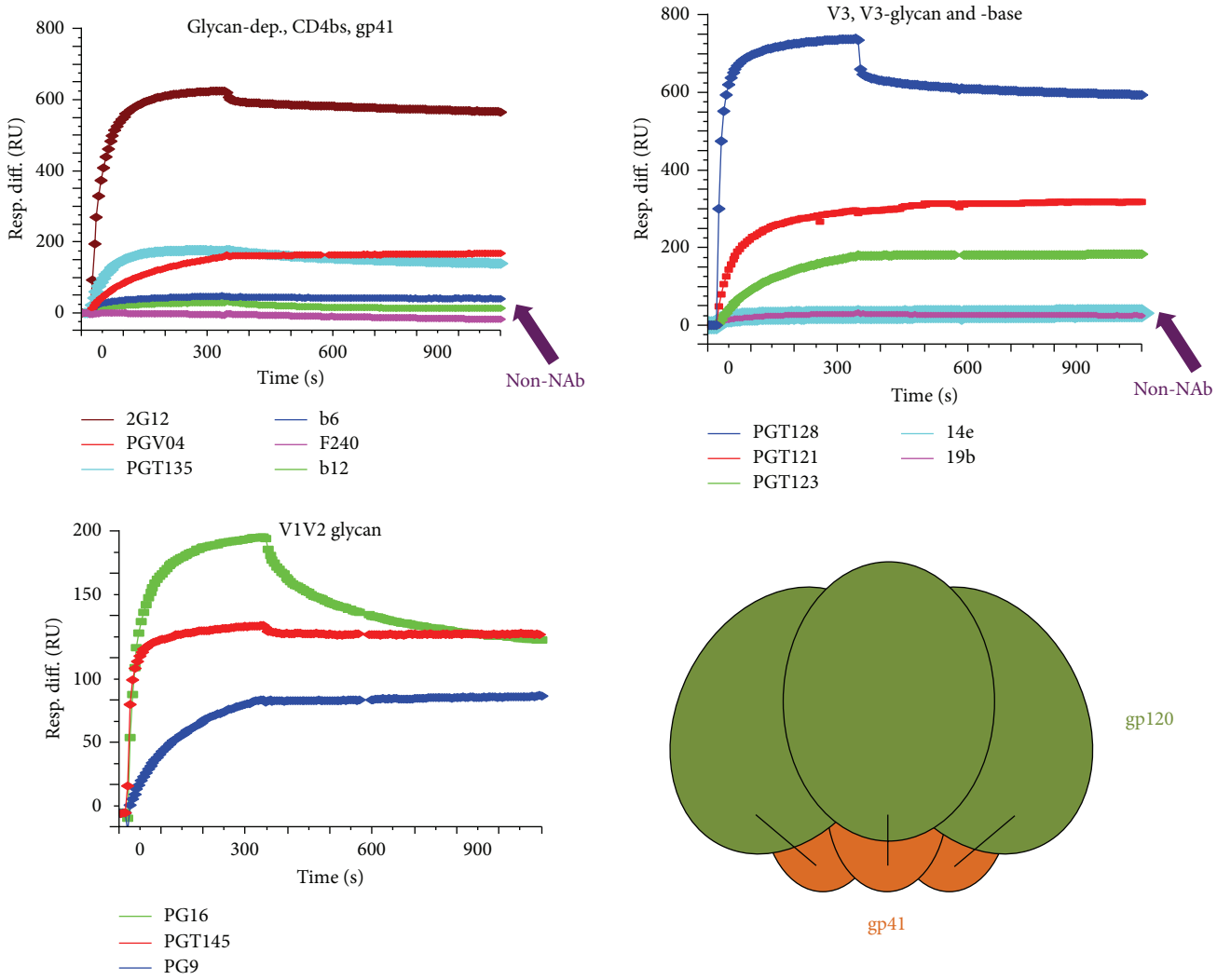


Figure 3: The kinetics of NAb binding. Soluble envelope glycoprotein oligomers can be immobilized on SPR chips via His or epitope tags. If the trimers are good structural mimics of native functional oligomers, and the density of trimers approximate that on the virion surface, then the NAb binding involved in neutralization can be simulated and its kinetic constants can be determined by SPR. Here, a soluble, stabilized trimer of the envelope glycoprotein of a Clade A isolate of HIV-1 was studied. The subunits of the trimer are labeled in the schematic to the lower right; the black bars represent engineered disulfide bonds that were introduced to stabilize each protomer of the trimer. The trimer was immobilized and the binding of NAbs and non-NAbs was compared. The sensorgrams show the response (RU) over time (s) during an association phase (upward curve) and a dissociation phase (downward curve: in several cases dissociation is very slow and barely measurable). Antibodies directed to different groups of epitopes, as indicated for the three diagrams, are compared. The nonneutralizing antibodies are marked with arrows. Thus, neutralization correlates eminently with binding to trimers that are native-like according to electron microscopy. The figure is reproduced from Sanders et al. [103] with modifications.

The outcome can differ greatly in accordance with such variations. Some NAbs gain substantially in efficacy when they are preincubated with the virus for prolonged periods. Drastic examples are NAbs directed to internal epitopes in the polivirus proteins VP1 and VP4 that are transiently exposed as the proteins "breathe" at physiological temperature [193]. Other NAbs appear to act after attachment of the virus to the target cells, even after receptor interactions [31, 99, 194]. If epitopes are transiently exposed only after receptor interactions, whole IgG molecules can be partially occluded from access to such sights in the multimolecular entry complexes; Fabs directed to such epitopes have been shown to be more potent than IgGs [195, 196].

Hence we can distinguish different modes of neutralization: induced decay in suspension and deceleration of entry at the cell surface or from an endosome. Strictly, mode is then distinct from mechanism, for the two modes may block the same replicative step, for example, receptor interactions. It is notable, however, that the TRIM21-dependent mechanism of neutralization of adenovirus and perhaps other naked viruses [142] would have a different relationship to the suggested two modes of neutralization: to the extent that the infectivity is reduced already in suspension or entry is delayed, the cytoplasmic action of the NAbs becomes redundant. But it might constitute an important third layer of protection, apparently the dominant one under specific circumstances

[143]. Intracytoplasmic inhibitory activities of NAbs would be a third mode.

The effects of NAbs acting on virions in suspension vary with epitope specificity and virus, and some of these effects are reversible, others irreversible [95, 160]. The virions on their own usually have a specific infectious half-life in suspension and the suspension mode of neutralization can be measured as the shortening of that half-life [194, 197]. As an example, for enveloped viruses with noncovalently linked subunits of the entry-mediating surface proteins, the NAb effect may be to induce the dissociation of an outer subunit so that the virus loses the capacity to bind to receptors; in the case of HIV-1, soluble forms of the main receptor, CD4, induce the dissociation of the gp120 subunit from the envelope glycoprotein spike. Such induced shedding, also mediated by some NAbs, may represent a premature triggering similar to, or more drastic than, what is induced by CD4 and coreceptor interactions at the cell surface or in an endosome in the absence of antibody during uninhibited entry [84, 132, 133, 198].

Thus, induced decay can be considered an aspect of the kinetics of neutralization: it would constitute a second inactivating step after NAb binding. Its relevance to protection *in vivo* will be contingent on prolonged periods of viral exposure to antibody before encounters with susceptible cells; that relevance would also depend on whether the antibody binding has nonneutralizing inhibitory effects such as opsonization of the viral particles for cellular destruction through phagocytosis. The latter might dominate. It should also be noted that the spontaneous as well as the induced decay may affect the number of entry-mediating molecules on the virion that must be inactivated by NAbs in order to block infection: an IC_{50} value of neutralization is not fixed but can vary with the preincubation time. Apparently, NAbs can nibble and chip away at the threshold of neutralization.

Although the binding of antibody is reversible, the deceleration of entry might have an irreversible outcome, as does the induced decay in suspension. By slowing down the productive entry, the NAbs may by default shunt the virus onto an abortive pathway [199]. Viruses that use penetration or fusion at the cell surface as an obligatory step could be routed onto an abortive endocytic pathway by Fc-Fc-receptor interactions or simply by delayed entry. That would seal the fate of the NAb-covered virion. The threat of lysosomal destruction lurks at the end of that route.

With viruses that use the endocytic pathway for entry the situation is different. For example, some NAbs bound to West Nile virus were reported to decelerate the internalization of the virus via the endocytic pathway and thus make the virus available for PAN longer [200]. Conversely, other NAbs, or at least lower occupancies of NAbs, can enhance infection of West Nile virus by favoring endocytosis and allowing fusion with the endosomal membrane [151, 201].

All other things being equal, any irreversible aspect of virus neutralization, whether through the induced-decay mode in suspension or the routing of virions towards destruction by cells in the other modes, must benefit host protection. Questions remain whether irreversibility of inactivation is redundant or crucial.

9. Molecularity: How Many Hits?

As we have seen, erroneous interpretations of neutralization kinetics have sown recalcitrant confusion about how many NAb molecules are required for neutralization, that is, its *molecularity,* the minimum number of molecules involved in the rate-determining step of a reaction. Kinetics of neutralization will not reveal its molecularity, but the kinetics of binding can give the affinity and hence allow legitimate estimates of occupancy. Occupancy is the equivalent of stoichiometry, and if the absolute number of functional, neutralization-relevant entry-mediating viral protein sites is known, the molecularity follows.

When the binding kinetics are studied by SPR, stoichiometric values can also be obtained, but these refer to the maximum binding of, for example, paratopes per oligomeric antigen; those data will be useful but not sufficient for correctly determining the molecularity at the level of the virion. And there are caveats to SPR determinations; for example, some NAbs dissociate so slowly that significant values of k_{off} cannot be obtained, or k_{on} is so high that mass-transport limitation yields uncertainty. Both affinity and stoichiometry, however, can also be determined by calorimetry, whereas stoichiometry in addition can be ascertained by electron microscopy (Figure 4) [103, 126, 182], and those data would provide necessary complements to and correctives of the SPR data.

Phenotypically mixed virus has been used in order to determine the molecularity of entry and neutralization more directly. Such virus preparations contain different proportions of antigenic and nonantigenic, or functional and defective, protomers of entry-mediating oligomeric proteins [111–114, 202]. Hence combinatorial mathematical equations, derived from the binomial theorem, can be applied to interpret the data [105, 107, 108, 110]. But also this approach entails pitfalls. If the entry protein is oligomeric and each virion, as is usual, has more than one oligomer, the modeling must simultaneously juggle two levels of analysis, each with its own potential threshold. One consequence is that quite different models can imply indistinguishable empirical data, because changes in the model premises at the oligomer and virion levels compensate each other [84, 105]. Notably, the premise that each virion only possesses a single entry-mediating oligomer, and therefore only needs one, translates into exactly the same equation as the most extreme incremental model mentioned before, namely, that each oligomer contributes equally to the infectivity, no matter how many intact oligomers are left per virion or how they are clustered.

The premises that infectivity is binary, all virions are fully or not at all infectious, and that there are no degrees of neutralization of the individual virion should probably both be modified. Already Andrewes and Elford came stumblingly close to predicating incremental neutralizing effects [203]: if their analysis had been heeded, some fallacies stemming from all-or-nothing premises would not have proliferated. Another extreme, that is, all unoccupied functional protein oligomers contribute incrementally in exact proportion to their number, may be equally unrealistic. A virion can probably be half-neutralized, but that does not mean that there cannot be

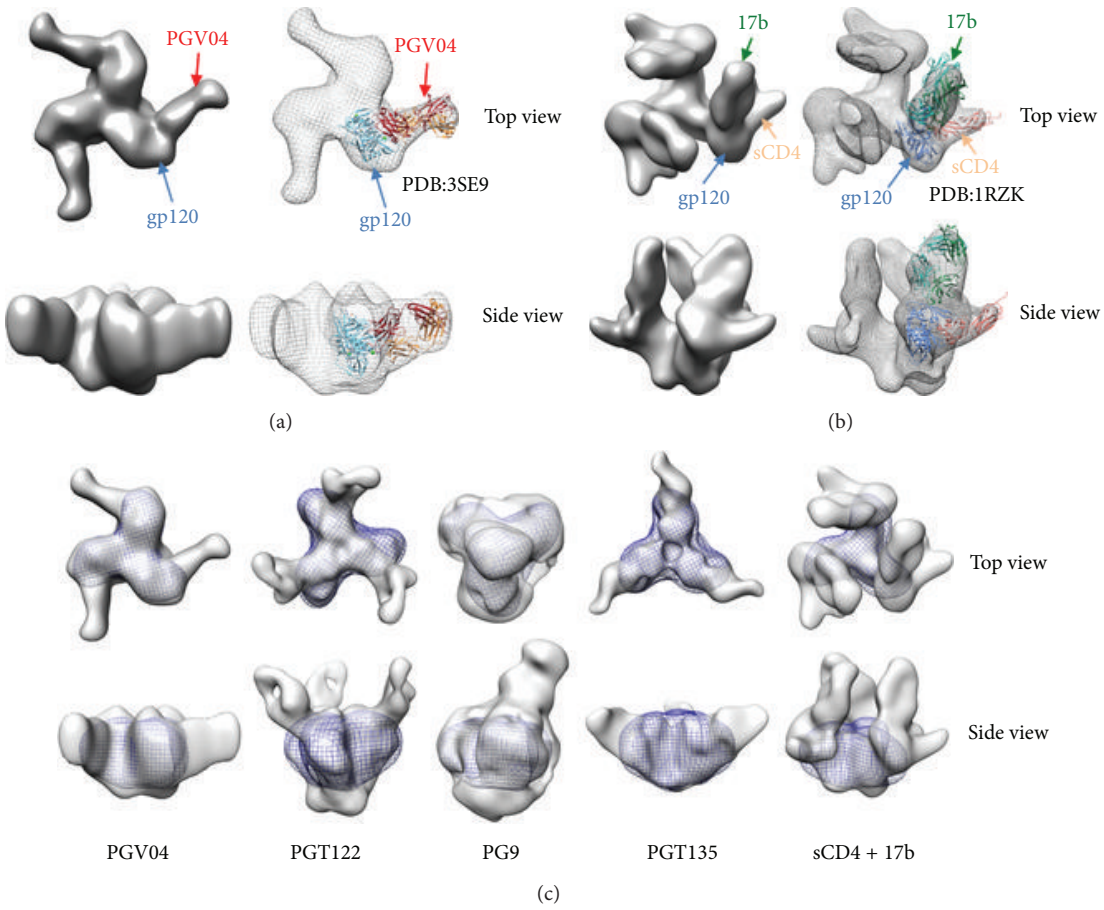


Figure 4: NAbs bind with different stoichiometries to oligomeric antigens. The images show electron-microscopy-based reconstructions of soluble trimers derived from the same Clade A isolate as in Figure 2 at ~23 Å resolution. (a) The propeller-like top view and the side view of the trimer with one Fab directed to the CD4-binding site (PGV04) on each of the three gp120 subunits. (b) The same trimer is shown in complex with a soluble form of CD4 and the Fab of an antibody that binds to the coreceptor-binding site (17b). This NAb neutralizes poorly as IgG and better as a Fab, because the epitope is not constitutively present and is relatively inaccessible in the viral entry complex when induced by CD4 interactions. Three copies each of CD4 and Fab molecules can be seen in the top view. (c) The same combinations as in (a) and (b) are shown first and last in the row. The second and fourth panels represent trimers in complex with the Fabs of two NAbs (PGT122 and PGT135) that bind to different epitopes and with different angles from each other and from PGV04 and 17b. In the middle is a NAb with the unusual stoichiometry of one Fab per trimer, PG9, which binds preferentially to trimers and at an oblique angle to an epitope with direct contributions from two subunits. The blue mesh delineates the trimer itself. The figure is reproduced from Sanders et al. [103].

redundancy in the number of entry proteins on virions or that all virions are proportionately infectious down to their last oligomeric spike or entry-mediating molecule [84, 105]. With these complexities in mind, one can nevertheless derive informative inferences from mixed phenotype data. And that is important because it helps estimating what concentrations and affinities of NAbs that vaccines must induce in order to protect.

Other approaches for determining neutralizing antibody occupancies are electron microscopy and various biochemical measurements of NAb binding to virions. The latter methods will only give an average degree of binding, but analogously neutralization assays merely provide data on the average infectivity for the virion population. Electron microscopy thus has an edge since it can describe the whole distribution of NAb occupancies over the virion population.

A source of artifacts with these binding assays collectively is that they do not distinguish between infectious and noninfectious virions. Knowledge of the ratio of infectious to noninfectious virions will be informative: the higher the ratio, the greater the relevance of the measurements. But as with neutralization, infectivity is not likely to be an all-or-nothing property.

Studies based on radioactively labeled NAbs indicated that poliovirus particles with one bivalently bound IgG molecule were neutralized [98]. Adenovirus was suggested to be fully neutralized with on average 1.4 NAb molecules bound per virion [204]. This raises questions of the precision or relevance of the measurements. Actually, neutralization and binding were not carried out under the same conditions in the latter study [204]: virus and NAb were incubated for 3 h at 37°C and then for an additional 21 h at 4°C before the

infectivity assay. The infectivity results were plotted as logarithmic survival as a function of linear serum concentration. In the binding analysis the NAb and virus were incubated for only 30 min at 37°C and then virion-NAb complexes were separated from free NAb by sucrose gradient centrifugation. The recovered virus was not infectious. Quite plausibly the separation procedure might perturb both equilibrium binding and virion infectivity. The analyses also pose theoretical problems.

A Poisson analysis was attempted to account for the binding-neutralization relationship under the two different conditions, the long and the short incubation. There are two problems. First, single-hit neutralization with Poisson-distributed binding and an average of 1.4 NAb molecules per virion implies a residual infectivity of about 25%. Since no infectivity was detected, either the virions were nonspecifically damaged by the procedure or the precision of the assay was insufficient for this kind of analysis. Second, the Poisson analysis of neutralization requires knowledge of the number of NAbs bound and the ensuing residual infectivity. The transition from a mere serum concentration to the multiplicity of bound NAbs would require much more information, namely, the average affinity and concentration [106, 205]. And the theoretical curve for logarithmic residual infectivity as a function of NAb concentration is then not linear even for single-hit neutralization [106, 205]. Only the curve postulating single-hit neutralization with average NAb multiplicity of binding on the x-axis is linear [205]. In practice, however, an approximately linear curve may very well arise if the lowest dilutions of the serum happen to yield NAb concentrations far above the average K_d value. Hence linearity observed under such conditions, contrary to the argument invoked in the study [204], is not evidence for single-hit molecularity.

The suggested single NAb binding to poliovirus mentioned above was not based on Poisson analysis [98]. A study that did apply Poisson analysis refuted single-hit neutralization of poliovirus and suggested a minimum of four NAbs per virion [206]. Notably, first-order kinetics were also observed, and it should be clear by now that there is nothing contradictory in those observations. This study derived the minimum number of NAb molecules from reading the value on the x-axis at $1/e$ relative infectivity. This procedure has been common practice but is not justified in theory. It is only for the single-hit curve that $1/e$ corresponds exactly to the minimum NAb number. The higher the actual number, the greater the deviation. Still, a molecularity of 70 IgG molecules per influenza virion was inferred by this method [158].

The Poisson analysis would provide the best test of the data by a comparison with the disparate theoretical curves for various molecularities: one, two, three, four, five, and so on hits [106, 205]. That way the correspondence of the data to a particular model can legitimately be assessed. Or at least the predicted relative infectivity for each model at the threshold value could be calculated and the readings could be compared with the theoretical value. With other molecularities than 1 (a minimum of one NAb per virion required for complete neutralization), the reading on the x-axis at $y = 1/e$ is as theoretically arbitrary as it would be at, say, 50% neutralization, which does not mean it is uninformative; at least it might refute a single-hit molecularity [31, 106].

For the record, Poisson analysis involves the following. The natural logarithm of the still infectious fraction of virus, $\ln(I/I_0)$, is plotted on the y-axis as a function of the average number of NAbs per virion, λ, on the x-axis. The minimum number of NAbs per virion required for neutralization is stipulated to have the integer value L. The infectious fraction of virions will be equal to the cumulated fractions with fewer than L NAbs bound to them: $I/I_0 = \sum_{r=0}^{L-1}(\lambda^r e^{-\lambda})/r!$. Thus with $L = 1$, the contested single-hit molecularity, and at an average of 1 NAb per virion, that is, $\lambda = 1$, $I/I_0 = e^{-1}$ so that $\ln(I/I_0) = -1$: if the single-hit hypothesis was true, approximately 37% of the infectivity would remain when the virions have on average one NAb bound to them. But obviously L will differ more from the x value at $y = 1/e$ the higher L is.

The prevalent errors in Poisson analyses may, however, dwarf in importance compared with the flawed premise of the approach: that all virions have equal infectivity in the absence of NAbs and that the threshold of neutralization is absolute; occupancies below it have zero effect; once it is reached neutralization is complete and higher occupancies make zero difference.

The reason for focusing on the pitfalls of inferences from and errors in the execution of Poisson analysis of neutralization is the great impact these flaws have had on the neutralization field. The single-hit molecularity has been elevated to virtual dogma on spurious grounds; it has been disseminated by textbooks [207]. The discovery of the TRIM21-dependent mechanism suggests that single-hit neutralization is molecularly plausible; it would then have been believed previously for the wrong reasons, and largely about the wrong viruses. In reality, the neutralization of adenovirus infection of mouse embryonic fibroblasts and of the human epithelial cell line HeLa occurred at average numbers of NAbs per virion of 1.6 and 4.8, respectively, when the NAb was murine. Both cell lines were IFN-stimulated to maximize TRIM21 expression. The explanation for the differing results between the cell lines would be that murine TRIM21 has a higher affinity for the murine NAbs than does human TRIM21 [143]. At lower TRIM21 levels much higher occupancies were required. Or at the extreme end, even saturating concentrations of NAbs yielded no measurable neutralization; likewise Fc mutants of NAbs, unable to interact with TRIM21, failed to neutralize. These new findings shed much light on the context-dependent and nonabsolute molecularities that are sufficient for neutralization. They also suggest a remarkable inefficiency of neutralization at steps before cytoplasmic entry. Therefore older observations with which they sometimes seem to clash should be revisited.

In an earlier study, adenovirus particles bound by NAbs failed to penetrate from the endosomal vesicle and would therefore not have been subject to TRIM21-mediated tagging for degradation; the observed block was at an earlier step. The suggested mechanism was that NAbs binding to the penton base prevented interactions with the endosomal membrane

and therefore blocked entry. Antifiber antibodies aggregated virions and thereby afforded some degree of neutralization. Antihexon NAbs blocked pH-induced conformational changes in the capsid thought to be conducive to penetration. Antihexon NAbs also mediated PAN [204].

Direct evidence supports multihit molecularities of neutralization of many viruses, not only enveloped ones. Thus 4-5 NAb molecules are required to neutralize poliovirus, one of the smallest viruses, 36–38 for papillomavirus, 70 for influenza virus, and 225 for rabies virus [99]. These ascending numbers suggest a roughly linear relationship between surface area of the virion and the minimum number of NAb molecules required for neutralization [99, 201]. This correlation agrees approximately with occupancy theories of neutralization but falls short of demonstrating their general validity. There are two naked viruses in the comparison but, as discussed, different principles apply to mechanisms and molecularities of the neutralization of naked and enveloped viruses. Hypothetically, the approximate linearity might apply best to enveloped viruses, but among them such factors as the density, functionality, and fragility of the envelope protein would play in; and host cell density can also determine the efficiency, that is, Nab concentration dependence, of neutralization [208]. In that regard, the observations are not based on comparable conditions for the different viruses. Therefore the rough correlation between virion size and the number bound NAbs required for neutralization is all the more striking.

The enveloped West Nile virus, which belongs to the flavivirus genus of the *Flaviridae* family, probably presents the quantitatively best understood example of antibody neutralization [149]. The same antibodies can both neutralize and enhance viral infection: which effect they have depends on the occupancy achieved. Around 120 epitopes for some particular NAbs are available per virion. When these NAbs occupy 25 of the epitopes, virion infectivity is reduced by half. At lower occupancies, these same antibodies are instead capable of enhancing infection by routing the virus onto the endocytic pathway via Fc receptors, thus without sterically blocking the ultimate fusion of the envelope with the endosomal membrane [151, 201].

Other NAbs both to West Nile and Dengue virus require higher occupancies on their epitopes for neutralization. The explanation, which elegantly illustrates the occupancy theory, is that fewer of those epitopes are accessible for NAb binding, so that to achieve a similar threshold number of NAbs or Fabs per virion, a greater proportion of the exposed epitopes must be ligated [149].

As mentioned, it has been suggested that low occupancies of the Env spikes on HIV-1 virions shunt the virus onto a nonproductive endocytic pathway, also through Fc-receptor interactions [148]. This mechanism of inactivation made sense when HIV-1 was thought to enter productively through direct fusion with the cell surface membrane. But now that strong evidence suggests the endocytic pathway is productive, and indeed even obligatory [83–85, 137, 209–211], this effect of the low antibody occupancies has become intriguing. Enhancing effects of low NAb occupancies on HIV-1 have also been observed [106]. From the vaccine perspective, it would be reassuring to know what different numbers of NAbs on virions do to the infectivity.

10. Extent, Efficacy, and Persistent Fraction

The persistent fraction (PF) of viral infectivity is the plateau of infectivity that is asymptotically approached as the incubation with NAbs is prolonged or the NAb concentration is increased (Figure 5). Here the focus will be on the latter, but some general remarks are warranted on the various hypotheses that have been formulated over many years to account for these phenomena [58, 99, 156, 184, 212–214].

A plausible explanation might be that the virus is heterogeneous and the PF simply represents a resistant variant. Although generalizations would be rash, at least in some cases, the virus which expanded from persistent fractions has, however, shown similar neutralization sensitivity to that of the original virus [58, 99, 184, 212, 214]. If genetically based causes are excluded, epigenetic ones, such as glycan-processing may be responsible for the persistence. If neutralization required conformational changes in the viral proteins, they might be induced to different degrees, and some virions might then be more resistant to the effect of NAb binding [58]. A spectrum of densities of functionally preserved neutralization antigen molecules over the virion population might also yield, if not absolute resistance, at least a tail of less sensitive virions.

Before PFs were demonstrated with monoclonal NAbs, heterogeneities among the antibodies in sera were implicated. The existence of neutralization-blocking antibodies was also invoked [58, 127]. Even monoclonal NAbs can be heterogeneous, because of variation in posttranslational modifications, for example, tyrosine sulfation that affects affinity [215]. Still, PFs are more general phenomena and their occurrence cannot be tied to particular NAbs although their sizes vary among NAbs.

Much focus of early PF research was on aggregates and how they would retain some infectivity and attach with greater avidity than single virions [58]. But PFs were also observed with monodispersed virus [213]. It should be noted that the law of mass action does not imply any PF (Figure 5): only when a subset of target molecules have aberrant, substantially reduced affinity would the neutralization tail off. Burnet fruitfully suggested that NAb dissociation is responsible for the PF [212]. This is compatible with the findings that both the addition of secondary antibodies, cross-linking those in the neutralizing serum, and the combination of NAbs to distinct epitopes can reduce the size of the PF [213]. The dissociation hypothesis gains further plausibility when applied to the dynamic competition between receptors and NAbs at the site of entry. It is also relevant that the degree of monovalent binding by IgG rises when binding approaches saturation, as expected from the well-established prozone effect [216]. Thus the avidity decreases and the competitive binding to receptors is favored over that to paratopes.

Recently, the PF of HIV-1 was shown to correlate with the off-rate constants of NAbs as measured with native HIV-1 Env trimers by SPR [183]. In addition, stoichiometry was

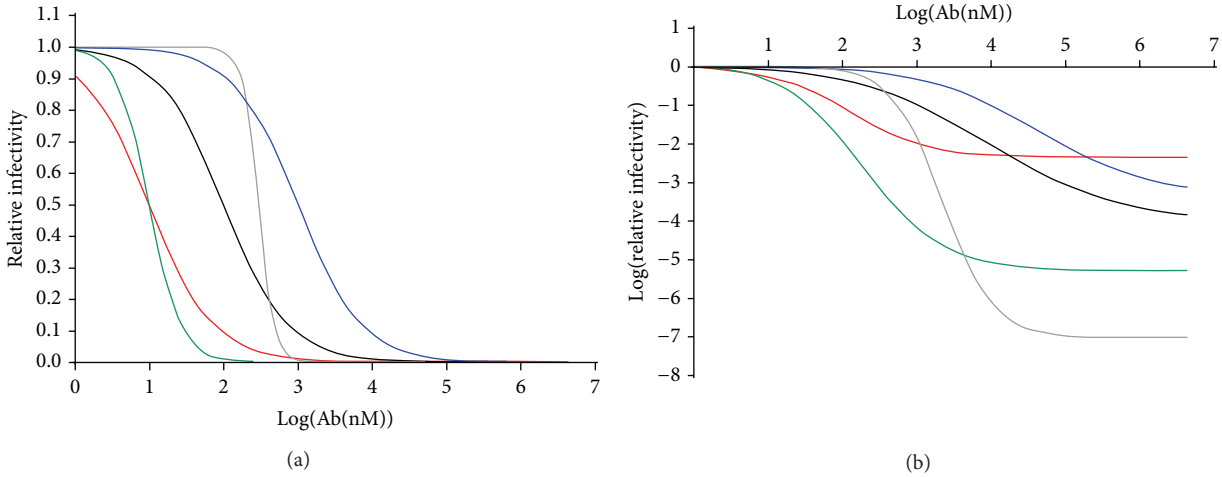


FIGURE 5: Neutralization potency and efficacy. (a) Potency: conventional neutralization curves (relative infectivity as a function of the logarithmic antibody concentration) for simulated data are shown. The green and red curves describe neutralization with identical potency, that is, IC_{50} values, although the green curve has a higher slope coefficient. Red, black, and blue curves represent decreasing potencies in that order while having the same slope. Neutralization represented by the grey curve falls between the black and blue in potency but has markedly higher slope coefficient than both. Antigenic heterogeneity reduces the slope coefficient and so does negative cooperativity. Positive cooperativity would raise the slope coefficient. (b) Efficacy: exactly the same simulated data as in (a) are plotted in a log-log plot to illustrate the importance of the *persistent fraction* (PF) of infectivity after neutralization. The two most potent NAbs from (a) (red and green) are shown to have widely different efficacies: the persistent fraction differs by about three logs. Furthermore, the curves for the less potent NAbs (black and blue) cross the red curve and tend towards a greater efficacy by one or half a log, respectively. The greater slope of the grey curve than of the others is apparent also in this plot but only here is the greater efficacy of the neutralization represented by the grey than by the green curve evident, another case of lower potency and greater efficacy.

related to the PF (Figures 4 and 5). Most NAbs to the HIV-1 Env trimer bind with a stoichiometry approaching three paratopes per trimer; but some trimer-specific NAbs only have a single epitope at the apex of the trimer (Figure 4) [175]. Thus stoichiometry as assessed both by calorimetry and SPR correlated with the size of the PF: the higher the stoichiometry, the lower the PF [183]. This finding might also be related to dissociation, because with a stoichiometry of one paratope per viral oligomer, the vulnerability to dissociation would be greater. Indeed, other evidence indicates that a single IgG molecule per trimer, of some NAbs, is sufficient to block the function of that trimer [84, 105, 108, 110, 111, 114, 202]. Thus when three are bound, two are redundant but would act as safety nets, particularly in the dynamic competition with receptors, when the demands on occupancy are highest.

Research into causes of the PF has been revived by the revelations about the TRIM21-dependent neutralization. Thus, neutralization of adenovirus by the same NAb leaves higher or lower PFs in inverse proportion to the levels of TRIM21 in the target cell [143]. But eventually a double plateau establishes itself: increasing NAb occupancies and TRIM21 expression levels reach diminishing returns, and the residual infectivity plateaus out. Indeed, the higher the viral dose is, the more easily the combined defense by NAbs and TRIM21 is overwhelmed. Quite plausibly, other cellular factors, those responsible for the degradation of NAb-virus complexes after the TRIM21-mediated ubiquitination, become limiting at this point. Clearly, the old problem of the PF has been greatly illuminated by the discovery of the role of auxiliary cellular factors in neutralization. The question is how far these insights could extend beyond adenovirus neutralization [142].

11. Synergy and Cooperativity

At the molecular level, two NAbs might potentially enhance each other's binding [217]. That would imply synergy in neutralization, benefitting the induction of antibody responses to multiple epitopes by vaccination. But synergy in neutralization has also been observed when there seems to be none at the level of antibody binding to the antigen molecule and indeed when there could not be any synergy in binding because the synergizing NAbs are directed to overlapping epitopes [218]. Then the explanation must be different. A plausible one is the heterogeneity in the population of target molecules, that is, the antigens, which can be extensive for some viruses [219]. This heterogeneity does not have to be genetically determined alone but could also stem from variation in posttranslational modification. Thus, synergy would arise from how two NAbs complement each other by covering different epitopes, or sometimes different variants of the same epitope, for which the NAbs have distinct affinities. This kind of explanation would apply equally to synergy in efficiency, that is, potency, as in efficacy, the latter being reflected in a reduced PF.

There have been conceptual as well as practical and modeling errors in synergy determination. But the classical synergy index, which contrary many claims does have general validity, can shed light on both synergistic and antagonistic

effects, provided it is calculated with approaches that avoid some prevalent sources of artifacts and errors [219, 220]. Still, it is purely a measure of synergy or antagonism in potency. Efficacy may be at least as important, not least *in vivo*, and will require an equally rigorous framework for analysis.

Cooperativity of single inhibitors acting on several sites on oligomeric target molecules has classically been measured as slopes, the Hill coefficients, of the inhibition curves [221]. It is clear, however, that heterogeneity can lower the slopes of those curves [219, 222, 223]. And that the combination of two NAbs with reduced slopes yields a higher slope may explain how antigenic heterogeneity gives rise to synergistic phenomena [219]. The slope of neutralization curves has been much less studied than the midpoint, that is, the potency, but it reflects an important property of the NAb and is partly the product of epitope heterogeneity; it can also be subject to paratope heterogeneity even for monoclonal antibodies when the intrinsic affinity is modulated by, for example, tyrosine sulfation, which will not be achieved uniformly over populations of NAbs produced naturally [215].

Generally, in studies of inhibition, when one molecule is inert and the other is active, but the combination is still more active, the proper term for the combinatorial effect is potentiation [224]. Regarding postentry neutralization it can be noted that interferons may potentiate neutralization by raising TRIM21 [141–143]. It remains to be seen to what extent the TRIM21-dependent inhibition merely provides a safety net for when NAbs fail to prevent entry, or whether it rather constitutes the main mechanism of neutralization, and against which naked viruses it is active. This field of study may present many new intricate problems of how cellular factors limit or enhance neutralization and whether the combinations yield additive, synergistic, or antagonistic net effects.

12. Conclusions: Reductionism Redux

It would be a great advantage in vaccine development to know that no other special feature is required of an antibody to render it neutralizing than simply its capacity to ligate functional entry-mediating viral proteins on the surface of the virion: vaccination becomes simpler if it is sufficient to create mimics of such viral proteins and make them immunogenic. For some viruses such as HIV-1 it is already very difficult to obtain sufficiently good mimics of the native Env protein and to focus the immune response on conserved regions, so that the antibodies induced will also recognize the Env trimers of naturally occurring divergent variants of the virus. Therefore any further complications, such as requirements for antibodies to induce particular conformational changes in the antigens or indeed elaborate posttranslational modifications required for the strongest binding, would be most unwelcome. Likewise, it is gratifying if neutralization works in such a way that antibodies capable of blocking it do not readily arise: it may still be impossible to avoid inducing some irrelevant, inert antibodies or, with some viruses, antibodies that enhance infection.

The comprehensive view of neutralization as interference with different steps of viral entry and as arising from a critical degree of coating of the virus particle with NAbs is supported by several lines of evidence for many viruses. But the discovery of the role of the cytoplasmic factor TRIM21 in adenovirus neutralization has provided an intriguing example of genuine postentry neutralization and rational explanations for why very few NAbs per virion are sometimes sufficient for neutralization. Whether this mechanism represents an exception or engenders a more general dichotomy between naked and enveloped viruses remains to be seen. Whenever naked viral cores can penetrate cell membranes while retaining bound NAbs, there is potential for intracytoplasmic neutralization expedited by cellular factors.

Improved biophysical characterization of NAb binding to native-like viral antigens provides much information of potential relevance to the efficacy of neutralization *in vitro* and protection by NAbs *in vivo*. The rough correlation between affinity and neutralization potency may, however, yield an oversimplified picture of the relationship between NAb binding and neutralization. Stoichiometry and kinetics of binding may affect also the efficacy of neutralization, which may be more important than potency for preventing the establishment of infection in an organism. Although improved knowledge of how NAbs protect would benefit vaccine development, any added requirements beyond sufficient binding to functional antigens would again make the task harder.

Early attempts to reduce neutralization to chemical principles were flawed, in part because infectivity and its inhibition were oversimplistically viewed as all-or-nothing phenomena. But now an emerging quantitative understanding of how viral proteins contribute to infectivity, together with the biophysical characterization of the binding of NAbs to viral antigens, may explain many aspects of virus neutralization. Although both the block of entry and postentry mechanisms need to be understood in their distinct molecular details, common principles may prevail, such as requisite occupancies and ubiquitous competition with the dynamic events of viral replication.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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