Viral Multiplicity of Attachment and Its Implications for Human Immunodeficiency Virus Therapies

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The multiplicity of attachment (MOA) of a virion in any particular time interval is the average number of cellular attachment opportunities that must be blocked to keep the virion in suspension. MOA is usually proportional to incubation time and cell concentration. Low MOA (like low multiplicity of infection) is required for reproducible assay of adsorptive blockers, and high MOA by itself can produce spurious synergies between adsorptive blockers, e.g., soluble CD4 (sCD4) and some antibodies. Poliovirus and human immunodeficiency virus (HIV) data show that viral neutralization conforms quantitatively to MOA and kinetic theory over large ranges of incubation times and target cell concentrations. Extrapolating sCD4 data beyond conditions achievable in vitro to those in vivo predicts that sCD4 concentrations above the strain-specific sCD4-gp120 dissociation constant are required to block lymphoid HIV significantly, in at least semiquantitative agreement with clinical results. The extrapolation is applicable to humoral neutralization data as well. MOA analysis also indicates that although completely stopping the attachment of individual virions to cells may still be an effective therapeutic strategy against established HIV infection, merely retarding attachment probably is not. The concept of MOA holds great promise for improving the therapeutic relevance of in vitro data and can be applied to any infectious agent, to many processes that impair or enhance infection steps, and to many assay end points, not just infection.

Successful infection requires a virus to attach to, penetrate, and uncoat and replicate within a host cell (5). Attachment is mediated by viral attachment proteins, present in multiple copies and unique to each virus type. Viral attachment usually requires the attachment protein to bind to a specific receptor site on target cells (48).

Many viral inhibitors are "antiadsorptives"; e.g., sulfated polysaccharides such as heparin, dextran sulfate, and suramin (55) and possibly some antibodies (15, 18, 43, 49, 50) block viral attachment. Soluble analogs of the cellular receptor are also antiadsorptives (6, 29, 38), although they sometimes have other antiviral effects (21, 26, 36). Of these analogs, soluble CD4 (sCD4), which is specific for the human immunodeficiency virus (HIV) attachment protein gp120, is one of the most extensively studied (10, 19, 23, 47, 52).

Because of the therapeutic implications, in vitro tests of any antiadsorptive should be reproducible and unambiguous. However, when different laboratories originally determined various neutralizing sCD4 concentrations against HIV-1_{IIIB}, the published results varied at least 500-fold, from 0.4 nM (6) to 200 nM (19). This inconsistency motivated the publication of a theoretical paper (32) predicting that high target cell concentrations could make antiadsorptives appear less effective.

The prediction took the virion's view of a neutralization assay. If an antiadsorptive impairs but does not permanently stop attachment of a virion, it might prevent the virion from attaching once, but at high target cell concentrations the virion may later encounter and infect other, alternative target cells. To prevent infection, the antiadsorptive must stop the virion more times at high target cell concentration than at low concentrations (30).

On the other hand, when the target cell concentration is

sufficiently low, the virion encounters at most one target cell during incubation. If the antiadsorptive stops the virion, the virion has no alternative targets and the assay registers one less infection. Certain controls (described below) are therefore necessary to ensure that an assay faithfully reflects individual antiadsorptive events (30).

Consider now a different, contrasting antiadsorptive, one that completely stops virions from attaching. Because rescue by alternative target cells is now impossible, increased target cell concentrations do not mask the effects of the blocker. Since specific, technical terms for expressing this distinction would be useful, I propose to use the terms "ablation" for the complete loss of viral attachment and "impairment" for partial losses. Viral neutralization therefore occurs through ablation or impairment of attachment or through inhibition of a postbinding infection step.

Note that the chemical mechanism (reversible or irreversible) of an antiadsorptive does not necessarily determine its effect on virions (impairment or ablation). For example, one antiadsorptive might bind irreversibly to virions but only impair their attachment to cells, whereas another may bind reversibly but be maintained at the surface of a virion in high enough concentration over a particular time interval (e.g., an assay time course or viral infectious lifetime in vivo) to stop cellular attachment completely and therefore to ablate the virion.

Distinguishing between impairment and ablation can aid experimental interpretation. For example, experiments have shown that high target cell concentrations do indeed rescue impaired virions. The blocking activity of sCD4 against HIV-1_{HXB3} decreased 20-fold as the target cell concentrations increased from 6.25×10^4 to 1.6×10^7 cells per ml (31).

This finding has immediate clinical relevance. The CD4⁺ cell concentration in a typical infectivity assay is about 10^6 cells per ml, approximating that in blood, whereas concentrations in lymph nodes are about 10^8 cells per ml. In vitro assays therefore systematically underestimate the potential lymphoid

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FIG. 1. Diagram comparing and contrasting MOA and MOI. The diagram shows four separate assays, with virions (small stippled circles) encountering target cells. The four assays correspond in relative terms to low MOI, low MOA (lower left); low MOI, high MOA (lower right); high MOI, low MOA (upper left); and high MOI, high MOA (upper right). Each arrow represents what a virion's path during its lifetime (or before washing) would be if attachment could not occur. Sometimes, under the prevailing assay conditions, the virion can in fact attach irreversibly (white squares); these are attachment opportunities. If each of the four imaginary assays were run several times, the MOA of a virion would be the average number of its attachment opportunities per assay run. Variables increasing MOI (assuming that other variables remain constant) are shown to the left of the MOI axis; analogous variables increasing MOA are shown to the right of the MOA axis. MOI and MOA have many other analogies. For example, a low MOI is required to make blocking assays reproducible and to avoid spurious synergies between viral blockers. When blockers impair viral attachment, the Theory section shows that reproducible blocking also requires a low MOA.

rescue of impaired virions (12, 31, 32). Because most of the viral burden in established HIV infection resides in the lymphoid tissue (41), lymphoid rescue probably contributes to the therapeutic failure of “neutralizing” concentrations of sCD4 (25, 46). It may also contribute to the lack of relationship between some types of antibody titers and clinical outcome (56) and degrade the efficacy of some immune therapies. (Differences between laboratory and wild-type strains of HIV [9, 39], emergence of escape mutants [28, 57], decrease in neutralization titers because of clinical immunosuppression, etc., may of course also contribute to these phenomena.)

A theoretical framework for anticipating the effects of target cell concentration (and virus-cell incubation time) on blocking would therefore significantly advance the preclinical assessment of potential therapies and vaccines. In addition, since physical measurements of viral attachment do not assess infectious virions directly, an infectivity protocol that can differentiate adsorptive impairment from other neutralizing activities would also be extremely useful. These objectives motivate the next section.

THEORY

This section adheres to a standard nomenclature for quantifying infection (16).

In this context, and consistent with original usage, multiplicity of infection (MOI) is the average number of virions

infecting a target cell (17) (Fig. 1). At sufficiently high MOI, target cells may be infected by several virions, so multiple infections may mask viral blocking. Viral blockers are therefore usually assayed at viral concentrations with a low MOI. MOI is a quantity associated with target cells. Consider instead the virions in an assay. Prevailing assay conditions give any specific virion certain “attachment opportunities” where the virion can attach, thereby committing itself irreversibly to a single target cell. Let us define the virion's multiplicity of attachment (MOA) in any time interval of interest (e.g., an assay time course or viral infectious lifetime *in vivo*) to be the average number of attachment opportunities that must be blocked to keep the virion in suspension (Fig. 1). (MOA quantifies the older, less definite notion of viral saturation [30–32].)

Mathematically, attachment opportunities can be modeled as a Poisson process (2) of the general type (13). In any particular assay run, for example, the actual number of attachment opportunities (that must be blocked in any particular time interval to keep a virion in suspension) is therefore Poisson distributed, with the mean equaling the MOA. If the MOA is denoted by m and the proportion of virions attaching is denoted by a (Fig. 2A),

$$a = 1 - e^{-m} \quad (1)$$

FIG. 2. (A) Theoretical plot of the proportion of virions attaching versus MOA. If the MOA is denoted by m , then by the Poisson distribution the proportion of virions attaching is $a = 1 - e^{-m}$. At low MOA, as the diagonal line indicates, attachment is proportional to the MOA. The horizontal line indicates that all virions attach. (B) Hypothetical experimental plots of the number of virions infecting versus virus-cell incubation time or target cell concentration. (Whenever the number of cells infected is used to measure the number of virions infecting, the assay must be run at low MOI.) Units on the axes have been omitted to emphasize that they differ from experiment to experiment. The circles represent the experimental control. At infinite incubation time or target cell concentration, the circles approach maximum infection, the top horizontal line. The squares represent the addition of a blocker that impairs virions by randomly blocking 50% of all attachment opportunities, which stretches the control graph to the right by twofold. Neutralization due to impairment therefore depends on the relevant x -axis variable, incubation time or target cell concentration. It is 50% at sufficiently small values of the x -axis variable but negligible at large values, where the squares approach maximum infection. The triangles represent the addition of a blocker that ablates 50% of all virions (i.e., completely stops them from attaching), which compresses the control graph vertically by 50%. A blocker inhibiting a postbinding infection step for 50% of all virions has the same effect. Neutralization due to ablation or postbinding inhibition is therefore independent of the x -axis variable, and at long incubation times or high target cell concentrations the triangles approach the bottom horizontal line, which is only 50% of the maximum infection. All the curves in panel B have a shape similar to that in panel A. The shape requires that infection be proportional to attachment, that infectious virions have similar rates of attachment, and that the integral in equation 2 be proportional to the relevant x -axis variable, either incubation time or cell concentration. The following common experimental conditions (among other possibilities) therefore degrade the curve fits implicit in panel B: heterogeneity of attachment rates; for plots of infectivity versus incubation time, decay of viral infectivity at long times and

Let us assume that a hypothetical antiadsorptive impairs a virion and randomly blocks 25% of its attachment opportunities, thereby reducing the virion's MOA by 25%. Because the curve in Fig. 2A is not linear, the inhibition observed depends on the original MOA. At sufficiently high MOA, the antiadsorptive must block the virion many times and appears completely ineffective. At low MOA, however, (as emphasized by the diagonal line in Fig. 2A) the number of virions attaching is proportional to MOA, so 25% impairment gives 25% observed neutralization. When viral attachment is impaired, reproducible neutralization assays therefore require a low MOA.

Ignoring MOA, like ignoring MOI, can also generate spurious synergies between blockers. For example, if two nonsynergistic antiadsorptives each reduce an original MOA of 16 down to 4 by impairing virions, together they reduce it from 16 down to 1. The corresponding effect on infection can be deduced from Fig. 2A: individually, the antiadsorptives have a negligible effect; together, they appear synergistic. By contrast, when the original MOA is low, each antiadsorptive substance reduces infection by a factor of 4, and the pseudo-synergy disappears. High MOA can therefore produce spurious antiadsorptive synergies.

Let k_a denote the rate of viral attachment; C , the target cell concentration; and T , the virus-cell incubation time. Then

$$m = \int_0^T k_a C dt = k_a C T \quad (2)$$

where the simpler right-hand expression holds whenever the rate of viral attachment and the cell concentration both remain constant. The legend to Fig. 2B lists some of the common experimental conditions, e.g., decay of viral infectivity at long incubation times, that may invalidate the simpler expression. Unless otherwise stated, the following will assume that the simpler expression is valid.

Kinetic theory (17, 42) usually combines equations 1 and 2, but equation 2 by itself has an attractive and useful physical interpretation: MOA, the average number of attachment opportunities, is proportional to the rate of attachment of a virion, the cell concentration, and the incubation time.

Many blocking assays involve incubating cells with virus, washing the blocker and unattached virions away from the cells, and then permitting the attached virions to infect. Under such protocols, infection is often proportional to the number of attached infectious virions (20, 22, 33, 34). When infectious virions are relatively homogeneous, infection is proportional to the y coordinate in Fig. 2A.

Barring specific experimental conditions (listed in the legend to Fig. 2B), plotting infectivity data gathered over a range of incubation times or target cell concentrations therefore yields a shape similar to Fig. 2A. When an experimental plot has this shape, curve fitting can extrapolate infection across incubation times or cell concentrations.

Different types of blocking leave different signatures on Fig. 2B. The effects shown require that the relevant x -axis variable (incubation time or cell concentration) not influence the blocking reaction. Experiments can often be constructed to satisfy this constraint (e.g., in incubation time experiments the blocker can be preequilibrated with virions before cells are added). Figure 2B then provides a powerful tool for dissecting blocking mechanisms.

variation of blocking or cell concentration over time; and for plots of infectivity versus cell concentration, toxicity at high cell concentrations.

RESULTS

Figure 3 displays previously published data (30, 33) and shows by curve-fitting that they conform quantitatively to Fig. 2. Figure 3 provides several checks on experimental technique. (Conclusions in the following, being based on infectivity data, are restricted to infectious virions.) In each experiment, the attachment rates for infectious virions probably lay within a fourfold range, since otherwise Fig. 2 and 3 could not agree quantitatively (see the legend to Fig. 2B). The agreement reflects experimental efforts to reduce viral heterogeneity. The same agreement indicates that viral decay during the poliovirus incubation and toxicity at high cell concentrations in the HIV experiment were not significant problems.

Extrapolating Fig. 3A to infinite time yields a maximum potential of 0.035 PFU/ μ l in the presence of hyperimmune serum and 4.6×10^4 PFU/ μ l in its absence (after multiplication by $10^{5.7}$ to account for dilution [see the legend to Fig. 3A]). Comparing Fig. 3A and 2B indicates that the rabbit hyperimmune serum caused ablation of poliovirions, or post-binding inhibition, or both. The corresponding fitted mean times to cellular attachment were 8 min in the presence of hyperimmune serum and 26 min in its absence (see the legend to Fig. 3). Rabbit hyperimmune serum therefore accelerated the attachment of infectious poliovirions. Historically, the acceleration was not concluded from these data but only later, from radiolabeling data (34). The radiolabeling data cannot, however, exclude the possibility that the accelerated attachment was restricted to a noninfectious subpopulation of poliovirions. The infectivity analysis can and does.

Curve-fitting to Fig. 3B and other, similar plots gave attachment rate constants for HIV-1_{MN}, HIV-1_{HXB3}, and HIV-2_{NIH2} of $k_a = 10^{-5.85} \pm 0.06$, $10^{-6.22} \pm 0.04$, and $10^{-6.6} \pm 0.1$ ml/h, respectively, in the absence of sCD4 (Fig. 4A). These results imply that, e.g., an infectious HIV-1_{MN} virion incubating in $10^{5.85} = 7.1 \times 10^5$ CEM-SS cells per ml has on average one attachment opportunity per h.

Figure 4 displays the parameters that gave the best fit to sCD4 blocking data for HIV-1_{MN}, HIV-1_{HXB3}, and HIV-2_{NIH2}. The sCD4 concentrations shown do not significantly accelerate gp120 shedding (30). The attachment rates k_a in Fig. 4A vary by only about twofold (0.3 log₁₀ unit), very probably within the error of the experiment and the curve fitting. More importantly, however, Fig. 4B shows that when infection is extrapolated to cell concentrations in lymph nodes, only sCD4 concentrations above the relevant strain-specific gp120-sCD4 dissociation constant reduced infection significantly. Although this threshold effect has been noted previously (30), extrapolation indicates that the threshold persists at cell concentrations in lymph nodes. The threshold may occur because successful HIV infection requires a virion to have a minimum density of free gp120 molecules (6, 30). If so, comparing Fig. 3B and 2B shows that subminimal gp120 densities do not cause impairment of attachment but, rather, ablation or postbinding inhibition or both.

DISCUSSION

Lymph nodes harbor most of the viral burden in established HIV infection (40). Because the high CD4⁺ cell concentrations in lymph nodes provide impaired virions with many extra chances for infection, they present unusual resistance to agents impairing viral attachment (11, 32). Hence, whenever a therapeutic agent (6, 7, 10, 19, 23, 43, 47, 52, 53) or serum (3, 45, 56) neutralizes (even potentially or partially) by impairing attachment, *in vitro* evaluation should explicitly consider the

FIG. 3. Plots of infection versus virus-cell incubation time and cell concentration from previously published data. The dotted lines are nonlinear least-squares fits to the data. In panel A, which shows the previously published poliovirus data (33), the fits are unweighted, whereas in panel B, which shows the previously published HIV-1_{MN} data (30), the vertical lines are error bars and the fits can thus be weighted accordingly. Fitting infectivity data to the equation implicit in Fig. 2B, $I = I_{\max}(1 - e^{-k_a C T})$, was done with routines programmed from the Numerical Recipes in C package (44). I is the number of infectious units (IU) per milliliter of stock, PFU for poliovirus or syncytium-forming units (SFU) for HIV. $I_{\max}$ is the maximum number of IU per milliliter, conceptually the number of IU at infinite incubation time or cell concentration if other biological conditions could be held constant. All curves pass a chi-square goodness-of-fit test to the corresponding points (44). In the poliovirus data, all variables are known except $I_{\max}$ and the lumped parameter $k_a C$, which are fitted to the data; in the HIV data, C is known and $T = 2$ h, so both $I_{\max}$ and k_a can be fitted. The text expresses poliovirus results as the mean time to viral attachment, $(k_a C)^{-1}$. Ablation and postbinding inhibition (Fig. 2B) reduce $I_{\max}$; impairment reduces k_a , slowing but not ablating viral attachment. (A) Plot of infection versus virus-cell incubation time for the previously published poliovirus data (33). The squares represent infection in the absence of rabbit hyperimmune serum, which, because of viral dilution in the experiment, should be multiplied by $10^{5.7}$. The circles represent undiluted infection in the presence of serum. (B) Plot of infection versus target cell concentration for the previously published HIV-1_{MN} data (30). The squares, circles, triangles, diamonds, inverted triangles, double triangles, and triangle-squares represent infection in the presence of sCD4 at concentrations of 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 nM, respectively.

FIG. 4. Plot of fitted values versus sCD4 concentration for the previously published HIV data (30). The legend to Fig. 3 describes the procedure for fitting data to the equation $I = I_{\max}(1 - e^{-k_a C T})$. The HIV data consist of Fig. 3B for HIV-1_{MN} and similar figures for HIV-1_{HXB3} and HIV-2_{NIH2} (not shown). The virus-cell incubation time for all HIV data was $T = 2$ h. The number of SFU per milliliter (I) was plotted against the cell concentration (C), and the fitted parameters, the maximum number of SFU per milliliter ($I_{\max}$) and the attachment rate (k_a), were then calculated. To compare the results for HIV-1_{MN}, HIV-1_{HXB3}, and HIV-2_{NIH2}, sCD4 concentrations (on the x axes) are given in units of the corresponding strain-specific gp120-sCD4 dissociation constant. For HIV-1_{MN}, HIV-1_{HXB3}, and HIV-2_{NIH2}, the constants are 0.6, 0.7, and 12 nM, respectively (30), all of which are represented for the corresponding curve by 1 on the x axis. The HIV-1_{MN} results are shown as circles joined by dashed lines; HIV-1_{HXB3} results are shown as squares joined by dotted lines; and the HIV-2_{NIH2} results are shown as triangles joined by chain-dashed lines. The curves have been jittered slightly to separate the points: the leftmost point in every curve actually corresponds to an sCD4 concentration of 0 nM. (A) Plot of k_a versus sCD4 concentration for the previously published HIV data (30). Increasing CEM-SS cell concentrations from 6.25×10^4 to 1.6×10^7 cells ml^{-1} can reduce sCD4 blocking activity against HIV-1_{HXB3} by 20-fold (Fig. 1b of reference 31), suggesting that low concentrations of sCD4 neutralize primarily by impairment, i.e., by reducing the attachment rate (k_a). However, this panel does not resolve any definite sCD4 influence on k_a , probably because the cell concentrations used in generating the curves were low, no more than 10^6 cells ml^{-1} (30). Although k_a , particularly for HIV-2_{NIH2}, shows some tendency to increase with sCD4 concentration, this acceleration is very probably within the error of the experiment and the curve fitting. Under the adsorption rates used, typical in vitro mean times to viral attachment are $(k_a C)^{-1} \approx (10^{-6} \text{ ml/h} \times 10^6 \text{ ml}^{-1})^{-1} = 1$ h. If the same rates apply in vivo, lymph node cell concentrations give a mean time to viral attachment of $(k_a C)^{-1} \approx [10^{-6} \text{ ml/h} \times 10^8 \text{ ml}^{-1}]^{-1} = 36$ s. Under such conditions, all infectious virions adsorb and the amount of infection then equals the

effect of cell concentrations in lymph nodes on blocking (30–32).

Whenever in vivo conditions are difficult to sustain in vitro, e.g., if cellular toxicity occurs in vitro at cell concentrations in lymph nodes, the only practical recourse is to extrapolate from achievable conditions. In this study a theoretical tool, MOA, has been developed to aid in quantitative extrapolations. Previously published data analyzed in the Results section show that the extrapolation is practical and that with careful experimental techniques it can be extraordinarily accurate.

Like MOI, MOA is a universal variable for quantifying virus-cell systems and describes how infection depends on virus-cell incubation times and target cell concentrations in many systems. Ignoring MOA, like ignoring MOI, leads to experimental artifacts and misinterpretations (30–32). For example, strain-specific gp120-CD4 affinities are known not to correlate closely with sCD4 neutralization of the corresponding HIV strain (1, 4, 54). One possible explanation for this seeming paradox follows (31).

By definition, the MOA in any particular time interval is the average number of attachment opportunities that must be blocked to keep a virion in suspension. Because the Results section shows that HIV strains can have different cellular attachment rates, an HIV-1_{MN} virion incubating in 10^6 CEM-SS cells per ml for 2 h must be blocked on average 2.8 times to prevent infection and an HIV-2_{NIH2} virion must be blocked 0.5 times (substitute k_a for HIV-1_{MN} and HIV-2_{NIH2} from the Results section into equation 2). Consider an sCD4 neutralization assay run under these conditions, and pretend for a moment (contrary to fact [24, 30, 35]) that the strain-specific gp120-CD4 affinities for HIV-1_{MN} and HIV-2_{NIH2} are the same. Despite equal affinities, sCD4 would neutralize HIV-1_{MN} less effectively than HIV-2_{NIH2}, simply because to prevent attachment, the assay conditions usually require sCD4 to block HIV-1_{MN} three times and HIV-2_{NIH2} only once.

Now reduce the cell concentration 10-fold to 10^5 cells per ml (or the incubation time to 0.2 h), so the MOA values become 0.28 and 0.05. Since sCD4 now usually has to block at most one attachment opportunity to keep either type of virion in suspension (30), equal gp120-sCD4 affinities now translate reproducibly into equal amounts of neutralization.

Higher attachment rates therefore enhance viral resistance to antiadsorptives at high but not low MOA. Although the relative sCD4 resistance of primary HIV isolates (8, 9) may be due to postbinding events (39), differing viral attachment rates may also be contributory (31).

Running assays at a low MOA can rule out this possibility (31). Since a low MOA (like a low MOI) generally enhances experimental sensitivity and reproducibility, equation 2 indicates that unless other considerations supervene, the use of brief virus-cell incubation times and low target-cell concentrations in a neutralization assay is generally a good strategy.

The following procedure determines in any particular assay

maximum number of SFUs per milliliter ($I_{\max}$), given in panel B. (B) Plot of the fitted value $\log_{10} I_{\max}$ versus sCD4 concentration for the previously published HIV data (30). To compare the results for HIV-1_{MN}, HIV-1_{HXB3}, and HIV-2_{NIH2}, $I_{\max}$ for each strain was normalized to 1 in the absence of sCD4. (The nonnormalized values of $I_{\max}$ in the absence of sCD4, representing the absolute infectivities of the stocks, were 120, 400, and 250 SFU/ml for HIV-1_{MN}, HIV-1_{HXB3}, and HIV-2_{NIH2}, respectively.) This panel shows that only sCD4 concentrations above the strain-specific sCD4-gp120 dissociation constant reduce $I_{\max}$ significantly. As explained in panel A, $I_{\max}$ corresponds theoretically to the amount of lymphoid infection.

the incubation times and cell concentrations producing a low MOA. With a fixed cell concentration, at sufficiently brief incubation times infection is proportional to the incubation time (Fig. 2B). Likewise, with a fixed incubation time, at sufficiently low target cell concentrations it is proportional to the cell concentration. In the range of proportionality (even if the rest of the experimental data do not resemble the control curve in Fig. 2B), a blocked virion does not get a second chance to attach (30). When the assay is run at incubation times and cell concentrations at or below the corresponding values in the range of proportionality, most virions have a low MOA. This primary MOA control has been put into practice (30, 31) and, like some control on MOI, is required to ensure reproducible results under adsorptive impairment for each new viral strain or target cell type (27).

Once the primary control is in hand, an easier, secondary control is possible in subsequent experiments if MOA influences neutralization by a particular blocker. For example, neutralization by low concentrations of sCD4 depends on the MOA, since increasing the CEM-SS cell concentrations from 6.25×10^4 to 1.6×10^7 cells ml^{-1} reduced sCD4 blocking activity against HIV-1_{HXB3} by 20-fold (31). If neutralization by such a blocker agrees with previous results obtained at low MOA, the assay MOA is probably still low (31). Equation 2 essentially enumerates the factors that can change the MOA. Since anticipating changes in attachment rates may be particularly difficult, some MOA control, either primary or secondary, is desirable even when the experimental conditions appear fixed. In one series of otherwise identical experiments, simply preincubating a single HIV-1_{IIIB} stock at 37°C for 4 h caused the measured sCD4 blocking activities to increase 20-fold, maybe partly because the viral decay during the preincubation decreased the attachment rates and hence the opportunities for viral attachment (31).

Neutralization by antiadsorptives in assays that cannot control virus-cell incubation times or target cell concentration (which includes many in use) may not be reproducible. If their MOA is high, their antiadsorptive results are being influenced by an important experimental variable of unknown magnitude (30–32).

High MOA, like high MOI, also leads to spurious antiadsorptive synergies (see the text explaining Fig. 2A). Nonneutralizing concentrations of anti-CD4 antibodies have been reported to enhance sCD4 neutralization of HIV (37). Neutralizing sCD4 concentrations in the absence of antibody were at least triple those in assays with a low MOA (30), however, suggesting that the assay in question had a high MOA and required sCD4 to block a virion several times to keep it in suspension. Similarly, two anti-gp120 antibodies, one against the CD4-binding site and the other against the V3 loop, have been reported to neutralize HIV synergistically (49). Both experiments also included enzyme-linked immunosorbent assay data directly demonstrating a binding synergy, but MOA controls on the infectivity assays are necessary to demonstrate that the viral neutralization was really synergistic in the first place (31).

Because of the available published data, this paper has presented MOA as a viral quantity. However, because it is a universal variable, MOA applies not only to HIV and poliovirus but also to other infectious agents; not only to sCD4 or antibody but also to practically any agent influencing viral attachment; not only to antiadsorptive but also to agents blocking other infection steps; and not only to assays measuring infection but also to other assay end points. In fact, (in physicists' parlance), MOA is pertinent to many situations in which particle attachment to a trap can be blocked. In the

attachment step of infection, the particle is the virus itself and the trap is a cell, but other particle-trap systems may present themselves elsewhere in the viral life cycle.

Let us consider, for example, the following hypothetical situation. An intracellular retrovirus (the particle) requires a single molecule of some nuclear factor called X (the trap) for insertion into DNA, and an assay counts the number of cells containing provirus in their DNA. The definition of MOA should replace attachment to a cell by attachment to a molecule of X; and the infection end point should be modified to insertion into DNA. The whole theory proceeds much as before: X is the target of the virion, the effective nuclear concentration of X is the target concentration, and cells with provirus replace infections as the end point plotted in Fig. 2B. (Equation 1 can even be modified if two molecules of X are required for viral insertion, just as MOI theory can be modified when infection requires viral cooperativity.) Extending MOA concepts beyond the viral attachment step will be experimentally challenging but not impossible.

MOA was known to depend on cell concentration (30), but its double proportionality to incubation time (see equation 2) greatly facilitates comparisons: e.g., increasing both incubation time and cell concentration 10-fold increases the attachment opportunities of a virion 100-fold. Since the double proportionality implies that incubation time and cell concentration have equivalent effects, it predicts that extending virus-cell incubation times, like increasing cell concentrations, will reduce inhibition caused by impaired attachment.

In vitro CD4⁺ cell concentration is often about 10^6 cells per ml; in lymph nodes, it is about 10^8 cells per ml. The HIV attachment rates in the Results section (Fig. 4A) show that an antiadsorptive substance must block an HIV-1_{MN} virion about once every hour in vitro and, unless attachment rates to CEM-SS cells and lymphocytes differ by orders of magnitude, that it must block the same virion about once every 36 s in a lymph node. In addition, however, typical in vitro incubation times are about 1 h; the spontaneous loss of HIV-1_{HXB3} infectivity (31) indicates an effective in vivo incubation time of about 10 h. Neutralizing a single virion in a lymph node by impairing its attachment is equivalent to the perfect neutralization of not tens, not hundreds, but thousands of equivalent virions in vitro. Impairing viral attachment is therefore very unlikely to play any role against established HIV infection (30–32), even as part of a combination therapy (51).

At sufficiently long incubation times or sufficiently high target cell concentrations, only ablation of attachment or postbinding inhibition provides effective neutralization (Fig. 2B). Thus, particularly for antibody neutralization tests (15), some attempt to define inhibitive mechanisms and to extrapolate in vitro inhibition quantitatively to the relevant in vivo conditions (3, 43, 45, 49, 56) (e.g., intravenous challenge with HIV, 10^6 to 10^8 cells per ml, ≈ 10 -h incubation time; or lymph node incubation with HIV, 10^8 cells per ml, ≈ 10 -h incubation time) should accompany any serious claim to clinical relevance.

Complete animal models of AIDS are rare and expensive (14), so accurate extrapolation from in vitro neutralization data assumes especial importance for HIV. The Results section extrapolated in vitro sCD4 neutralization data to lymph node conditions, indicating that only sCD4 concentrations exceeding the strain-specific gp120-sCD4 dissociation constant are likely to have a significant effect on lymphoid HIV. This extrapolation is consistent with clinical findings, perhaps even quantitatively so, since only patients receiving the highest sCD4 dose (30 mg daily) showed any decrease in their HIV antigen levels (25, 46). MOA theory, by accounting for the systematic effects of cell concentration and incubation time on

blocking, may therefore be able to raise the concordance between some in vitro and in vivo results to quantitative accuracies.

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