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Blocking of Human Immunodeficiency Virus Infection Depends on Cell Density and Viral Stock Age

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Quantitative infectivity assays were used to study how the blocking activity of soluble CD4 (sCD4) is affected by sCD4 concentration, target cell density, and viral stock age. During incubation with 20 nM sCD4, human immunodeficiency virus type 1 (HIV-1) stocks underwent irreversible inactivation. In contrast, inactivation with 2 nM sCD4 was almost entirely reversible. At lower sCD4 concentrations (≤2 nM) and target cell densities of 6.25×10^4 ml^{-1}, sCD4 blocking activity for HIV-1 gave a gp120-sCD4 association constant (K_{assoc}) of 1.7×10^9 M^{-1}, which agrees with chemical measurements. At the higher density of 1.6×10^7 cells ml^{-1}, however, the blocking activity was 20-fold less. During incubation of HIV-1 stock optimized for infectivity by rapid harvest, sCD4 blocking activity increased 20-fold during a 3-h window. These results show that competitive blocking activity depends strongly on target cell density and virion age. Thus, unappreciated variations in HIV stocks and assay conditions may hinder comparisons of blockers from laboratory to laboratory, and the age of HIV challenge stocks may influence studies of drug and vaccine efficacy. The results also suggest that blocking of viral particles in lymphoid compartments will require very high competitive blocker concentrations, which may explain the refractory outcomes from sCD4-based drug trials in humans.

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) share two properties that are important for understanding immunity and therapy. First, HIV preferentially infects CD4$^+$ target cells (9, 29, 37, 41), which have a wide range of densities in lymphoid, reticuloendothelial, and nervous tissues. For example, typical CD4$^+$ cell densities in blood and lymph node are 10^6 to 10^7 and 10^7 to 10^9 ml^{-1}, respectively. Second, the density of glycoprotein knobs covering the surface of HIV particles decreases with time. This spontaneous shedding occurs because the gp120 surface and gp41 transmembrane glycoproteins are associated by noncovalent interactions (30, 39). After observing this gp120 loss by electron microscopy, Gelderblom et al. (13, 14) suggested that knob density might influence the biological properties (e.g., infection and blocking) of HIV.

To address this hypothesis, we previously used a quantitative infectivity assay to determine how the binding of soluble CD4 (sCD4) (3, 6, 10, 12, 22, 58, 61, 64) to gp120 inhibited viral infectivity (32, 34). At low sCD4 concentrations, the inhibition of HIV-1 and -2 infection was proportional to binding. At slightly higher concentrations, there was a positive synergy in the inhibition of infection. One possible explanation for this concentration-dependent blocking activity was that HIV required a critical density of free gp120 glycoproteins for efficient infection of CD4$^+$ cells. When more than this critical density was present on HIV, infection proceeded at a rate proportional to free gp120 glycoproteins. In agreement with these findings, McKeating et al. (42) demonstrated a connection between the spontaneous shedding of gp120 and the loss of HIV-1 infectivity. Moore et al. (45, 46) have also shown that preincubation of HIV-1 stocks with high sCD4 concentrations (>5 nM) facilitated rapid shedding of gp120 that was accompanied by loss of infectivity. This facilitation was not observed at low sCD4 concentrations (<5 nM). These studies clearly demonstrated a relationship between gp120 and HIV infectivity. They did not, however, reveal the effects of target cell density on infection and blocking.

We therefore undertook an investigation of how sCD4 blocking activity depends on target cell density, viral stock age, and sCD4 concentration. sCD4 was selected as a model blocking agent for three reasons. First, chemical measurements of the gp120-sCD4 association constant (K_{assoc}) have been published by several groups (44, 58). As previously described (32, 34, 59), such measurements allow correlations between biological activity and the thermodynamic properties of the binding reaction. Second, crystallographic (55, 63) and biological studies indicate that CD4 has one high-affinity binding region for gp120 involving the CDR2 (7, 31, 43) and CDR3 (18, 35, 49) domains. This interaction represents a great simplification over the complexities inherent in multivalent blockers, such as immunoglobulins. Third, two large sCD4-based drug trials in humans are now under way, and to date only refractory outcomes have been reported (8, 25, 56). By examining sCD4 blocking activity with assay conditions mimicking physiologic ones (e.g., a range of target cell densities), we expected to gain some insight into the clinical outcomes.

Understanding how assay conditions influence sCD4 blocking activity has applications to investigations of other gp120 blocking agents and neutralization domains. Examples of such blockers include CD4 fragments (35, 49), CD4-immunoglobulin conjugates (2, 4, 62), monoclonal immunoglobulins (20, 27, 38, 57, 60), and some vaccine-induced immunoglobulins (1). The other blocking domains on gp120 include the third variable region (V3 loop) (15, 23, 24, 52–54), conformational epitopes (16, 17, 20, 50), and conserved sequences (5, 19, 21).

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MATERIALS AND METHODS

Quantitative infectivity assays. Infectious events were quantified by a modified version of the syncytium-forming viral infectivity assay (32, 47, 48) that minimized artifacts associated with high cell density. Before the assay, CEM-SS cells were grown at densities ($<5 \times 10^5$ ml^{-1}) to ensure their exponential growth. On the day of an assay, cells were suspended in fresh medium to serve as target and indicator cells. Target cells (5×10^5) were transferred to tubes containing four different volumes of fresh media and seven different sCD4 concentrations: 0, 0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 nM. The final target cell densities in the tubes were 1.6×10^7, 4×10^6, 1×10^6, and 6.25×10^4 ml^{-1}. The reaction volumes were 3.13×10^{-2}, 1.25×10^{-1}, 5×10^{-1}, and 8 ml, respectively. Graded volumes of HIV-1HXB3 stock were then added to each tube, resulting in a constant inoculum-to-volume ratio of 10%. To ensure uniform mixing, tubes were rolled during the 1-h infection period at 37°C. Next, the infected target cells were washed once (sufficient to remove sCD4 and cell-free virus) and suspended in fresh media at 5×10^4 cells per ml. Cell monolayers were prepared by adding 5×10^3 target cells and 3.5×10^4 indicator cells to flat-bottom microtiter wells. A total of eight wells were plated per sCD4 and target cell concentration. Syncytia, representing the infection of individual target cells by cell-free virus (47, 48), were counted on day 4 following plating. For the assays in Fig. 2, conditions were modified so the target cell density was 2×10^6 ml^{-1}, the reaction volume was 1 ml, and inoculum-to-volume ratio was 20% in all tubes. Thus, for the experimental stock, 1.4 and 0.28 nM sCD4 were present during incubation and assay, respectively. Cell monolayers were prepared by adding 1×10^4 target cells and 4.5×10^4 indicator cells to flat-bottom microtiter wells. For the assays in Fig. 3, conditions were modified so the target cell density was 5×10^5 ml^{-1}, the reaction volume was 1 ml, and inoculum-to-volume ratio was 20% in all tubes. The six sCD4 concentrations were 0, 0.4, 0.8, 1.2, 1.6, and 2.0 nM. Cell monolayers were prepared by adding 2.5×10^3 target cells and 3.5×10^4 indicator cells to flat-bottom microtiter wells.

Viral stocks. After H9 cells were inoculated at a multiplicity of infection equaling 0.1, the titer of infectious HIV-1HXB3 virions increased exponentially for 2 to 3 days (33). Hence, this multiplicity of infection was chosen for the preparation of optimized viral stocks. Before infection, H9 cells were grown at densities ($<5 \times 10^5$ ml^{-1}) to ensure their exponential growth. H9 cells were treated with DEAE-dextran (25 μg ml^{-1} for 30 min followed by centrifugation for 10 min at $200 \times g$) and incubated with virus for 1 h. The infected H9 cells were washed twice (centrifugation for 10 min at $200 \times g$, suspension in phosphate-buffered saline, and another centrifugation), suspended in fresh medium (5×10^5 cells ml^{-1}), and incubated with mixing at 37°C. Two days later, the infected H9 culture was clarified by centrifugation (20 min, $10{,}000 \times g$) and placed in an incubator. Infectivity assays were conducted either immediately following clarification or after specified intervals of incubation with mixing at 37°C.

Soluble gp120 in viral stocks. Typically, after ultracentrifugation of day 2 HIV-1HXB3 stocks (40 min, $155{,}000 \times g$), the concentration of soluble gp120 in the supernatant was $\leq 5 \times 10^{-10}$ M by gp120 enzyme-linked immunosorbent assay (ELISA) (American Bio-Technologies). Thus, for inoculum-to-volume ratios of ≤20%, the average concentrations of soluble gp120 were at least sixfold smaller than $1/K_{assoc}$. For all infectivity assays, this ensured that soluble gp120 did not compete significantly (32) with virus-associated gp120 for sCD4 or cell surface CD4 during quantitative infectivity assays.

TABLE 1. Classifying the blocking activity of sCD4[a]

sCD4 concn (nM)	SFU/ml (mean ± SEM)[b]	Irreversible inactivation of HIV (fold)
0	$(1.26 \pm 0.01) \times 10^4$	None
2	$(1.15 \pm 0.02) \times 10^4$	1.1
20	$(2.0 \pm 0.2) \times 10^2$	63

[a] Day 2 viral stock was used.
[b] Results are averages of data for 16 microtiter wells for each sCD4 concentration.

Removing sCD4 from preincubated viral stocks. To assess sCD4 blocking activity, 8-ml aliquots of HIV-1HXB3 stock were preincubated with 0, 2, and 20 nM sCD4 for 80 min at 37°C. sCD4 was then reduced 1,000-fold by ultracentrifuging the preincubated stock (40 min, $155{,}000 \times g$), removing the supernatant, and suspending the pellet in 8 ml of fresh medium. The numbers of syncytium-forming units (SFU) remaining after these manipulations are shown in Table 1.

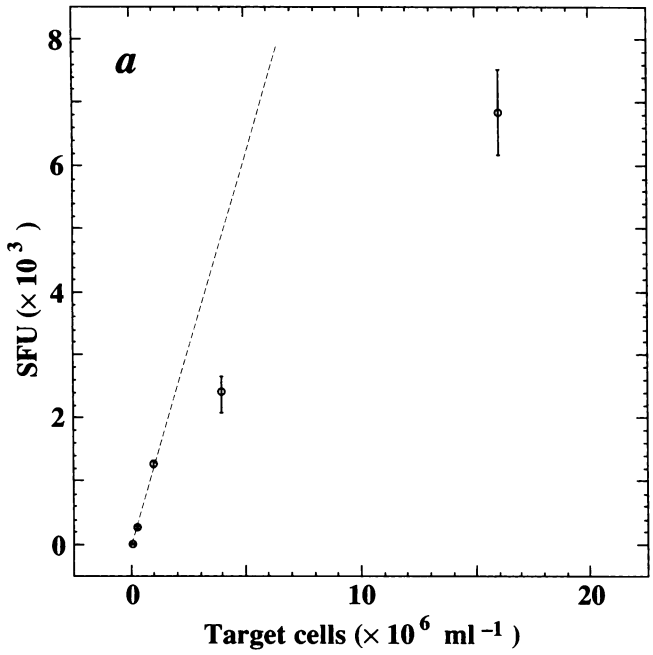
Quantifying blocking activity. To assess the inhibitory effects of sCD4 on HIV infectivity, plots of normalized inverse infection, 1/SFU with blocker multiplied by SFU without blocker, versus sCD4 concentration were constructed (32, 34). For unsaturated and saturated infectivity assays, the slope of such plots, [normalized inverse infection − 1]/[sCD4], is the molar blocking activity. This definition applies to each concentration of blocker and does not require that inverse infection plots be linear.

Statistical analysis. Minimizing $f(a, b) = \Sigma\{[y_i - (ax_i + b)]/\sigma_i\}^2$ gives a weighted least-squares linear fit to the data (26). y_i is the mean value of 1/SFU at the ith sCD4 concentration, σ_i is the standard deviation of 1/SFU, and x_i is the sCD4 concentration at the ith data point. For normalized inverse infection plots, the weighted least-squares fit gives slope = a/b. In Fig. 1b and 3a, weighted least-squares linear fits were calculated from all data. In Fig. 3b, because of upward curvature, fits were calculated from data at the lower four sCD4 concentrations. In Fig. 1a, the unweighted least-squares fit was obtained by setting $\sigma_i = 1$ at the lower three target cell densities. Confidence limits for the blocking activities in Table 2 were calculated by a standard bootstrap method (11).

RESULTS

Reversibility of the blocking activity of sCD4. Agents that block HIV by binding to gp120 are either reversible after removal (competitive) or irreversible (noncompetitive). To evaluate sCD4 with respect to these categories, we preincubated HIV stocks with increasing concentrations of blocker (Table 1). After removal of sCD4 from these stocks, the number of infectious units was assessed with a quantitative infectivity assay. Compared with the control, 2 and 20 nM sCD4 inactivated HIV in 2 h by 1.1- and 63-fold, respectively. Thus, at high concentrations, sCD4 caused irreversible viral inactivation, as already reported by Kirsh et al. (28) and Moore et al. (45, 46). At low concentrations, however, sCD4 blocked HIV competitively.

Effects of assay saturation on competitive blocking activity. It has been well established that HIV particles lose their infectivity with time (36, 40, 42). Assaying the infectivity of virions therefore reflects the race between spontaneous viral



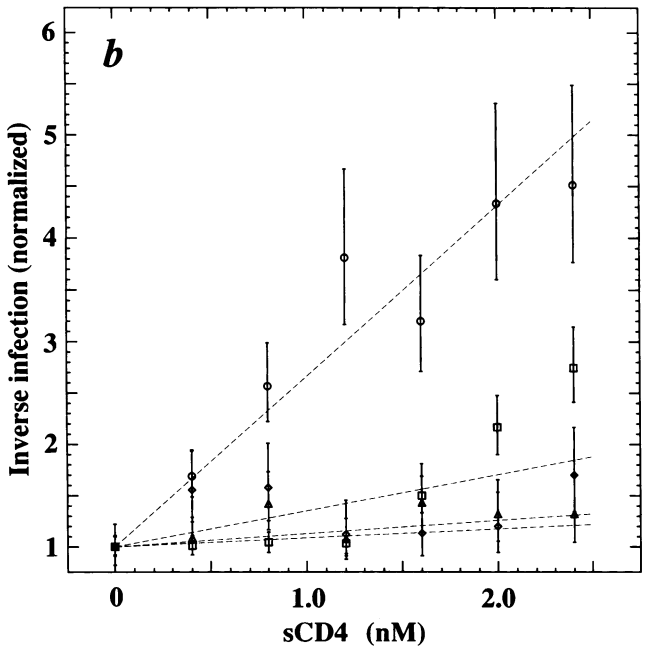


FIG. 1. Measurement of sCD4 blocking activity. (a) Detection of assay saturation in the absence of blocker. The figure plots SFU versus target cell (CEM-SS) density for assays without sCD4. For unsaturated assays, SFU are proportional to target cell density (32). As assays become saturated, SFU are less than proportional to target cell density (34, 59). The dotted line is an unweighted least-squares fit to data at the lower three target cell densities. (b) Measurement of infectious events in the presence of blocker. The figure plots normalized inverse infection, 1/SFU with blocker multiplied by SFU without blocker, versus sCD4 concentration. The slope, [(normalized inverse infection)−1]/[sCD4], is the molar blocking activity. The figure shows results at target cell densities of 6.25×10^4 (○), 1×10^6 (□), 4×10^6 (△), and 1.6×10^7 (◇) ml^{-1}. Dotted lines are weighted least-squares fits to the data. Results are means of eight microtiter wells (bars show ± 1 standard deviation). As target cell densities increase, the assays become saturated and the blocking activity decreases.

TABLE 2. Summary of sCD4 blocking activity

Target cell density (ml^{-1})	Viral stock	Incubation (h)	Blocking activity[a] ± 1 SD (M^{-1})	Blocking activity ratio
Fig. 1b				
6.25×10^4	Day 2	0	$(1.7 \pm 0.1) \times 10^9$	1.0
1.00×10^6			$(3.5 \pm 1.0) \times 10^8$	0.21
4.00×10^6			$(1.3 \pm 0.3) \times 10^8$	0.077
1.60×10^7			$(8.5 \pm 7.0) \times 10^7$	0.050
Fig. 3a				
5.00×10^5	Day 2	0.5	$(1.0 + 0.1) \times 10^8$	1.0
		6	$(3.3 \pm 0.1) \times 10^8$	3.3
		13	$(8.4 \pm 0.2) \times 10^8$	8.4
Fig. 3b				
5.00×10^5	Day 6	0.5	$(1.8 \pm 0.2) \times 10^8$	1.0
		6	$(5.4 \pm 0.5) \times 10^8$	2.3
		13	$(1.7 \pm 0.2) \times 10^9$	9.4

[a] Using strain HIV-1HXB3 and the same formulation of sCD4, previous measurements of the gp120s-sCD4 K_{assoc} yielded $(1.4 \pm 0.2) \times 10^9$ M^{-1} from unsaturated infectivity assays (32).

inactivation and target cell infection. Figure 1a plots SFU versus target cell density for quantitative assays of HIV-1HXB3. At low target cell densities, every virion has at most a single chance to infect before inactivation. In such unsaturated assays, blockers will perturb single infectious events. At higher target cell densities, virions may have several chances to infect before inactivation. In such saturated assays, blockers must inhibit several potential infectious events. Hence, we expected that competitive blockers would appear significantly less effective at high target cell densities (32, 59).

To test this hypothesis, we conducted quantitative infectivity assays at low sCD4 concentrations and for a range of target cell densities. In unsaturated assays and at low sCD4 concentrations, previous work (32) showed that sCD4 blocking activity (defined in Materials and Methods) gave the gp120-sCD4 K_{assoc}. In this study, when the target cell density was 6.25×10^4 ml^{-1}, sCD4 blocking activity of 1.7×10^9 M^{-1} (Table 2) agreed with previous biological (32) and chemical (44, 58) measurements of K_{assoc}. As target cell densities increase, however, the blocking activity of sCD4 declined progressively with assay saturation (Fig. 1b). At a target cell density of 1.6×10^7 ml^{-1}, the blocking activity declined 20-fold (Table 2). Target cell density therefore directly influenced competitive blocking activity to HIV.

Effects of viral stock age on competitive blocking activity. Figure 2a shows the spontaneous inactivation curve for a single HIV-1HXB3 stock at 37°C that was rapidly grown and harvested at optimal infectivity. The lower curve shows inactivation in the presence of 1.4 nM sCD4, while the upper curve is the control. Both curves show a 2-h transient phase followed by a initial phase of slow inactivation lasting 6 h. This phase is then followed by a pronounced shoulder, suggesting multihit inactivation. This pattern occurred with either CEM-SS cells (47, 48) or freshly isolated human peripheral blood lymphocytes, and both target cell types measured similar losses of SFU or 50% inhibitory dose over time (33). During the initial phase, 1.4 nM sCD4 did not measurably increase the rate of inactivation compared with the control. During the final phase, however, a marked increase in the rate of inactivation occurred in the presence of sCD4. To determine whether other soluble factors like

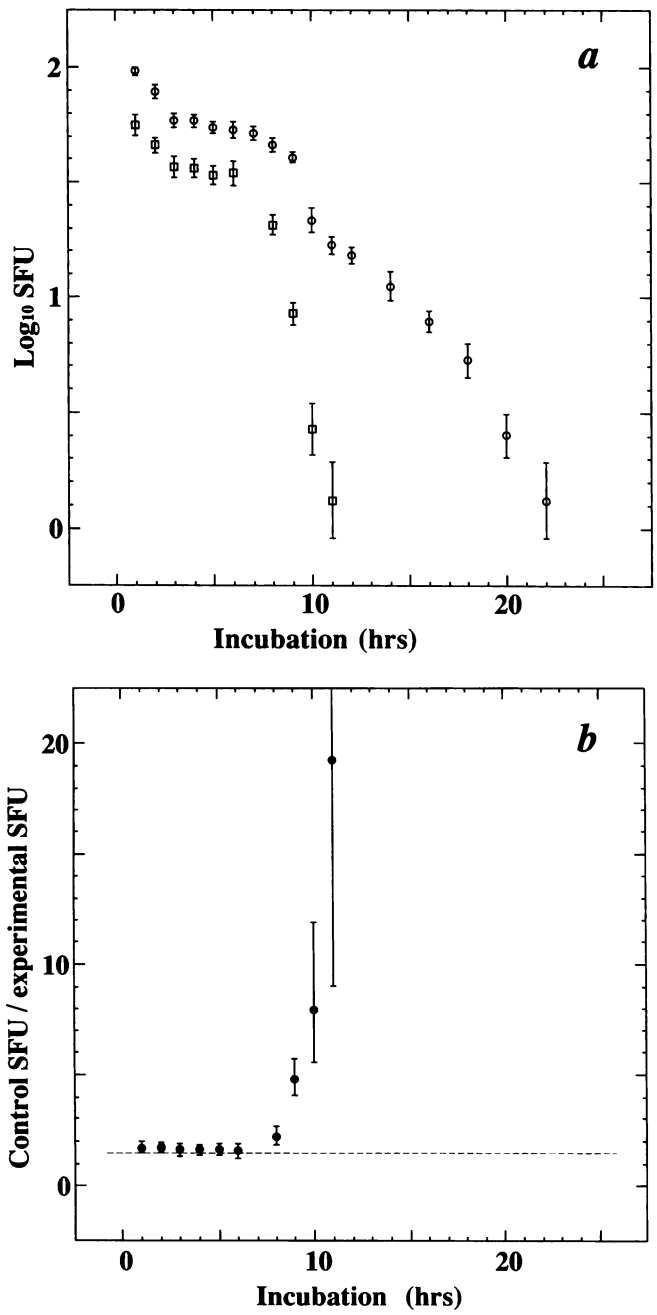


FIG. 2. Measurement of sCD4 blocking activity as viral stocks spontaneously inactivate. After 2 days of exponential viral replication, equal volumes of clarified HIV-1HXB3 stock were added to separate flasks. To the experimental stock, blocker in medium was added to obtain 1.4 nM sCD4; to the control stock, an equal volume of medium without blocker was added. Afterwards, both flasks were incubated at 37°C with gentle mixing. At hourly intervals, the number of SFU in the experimental (□) and control (○) stocks were measured by using an unsaturated infectivity assay (a). Results are the geometric means of eight microtiter wells (bars show ± 1 standard deviation). (b) Ratio of control SFU divided by experimental SFU (bars show ± 1 standard deviation). The dotted line is the expected ratio of control to experimental stocks, based on assay conditions and a gp120-sCD4 K_{assoc} of 1.7×10^9 M^{-1}. During the first 8 h, sCD4 blocking remained constant ($1.5 \leq$ ordinate ≤ 2.3). Thereafter, over 3 h, the blocking activity of sCD4 increased by more than 20-fold.

proteases and surface-active agents contributed to degradation, the viral stock was diluted fivefold with fresh medium and incubated at 37°C. Dilution did not affect inactivation (33), ruling out significant contributions by soluble factors.

Figure 2b shows the ratio of SFU in the control and experimental stocks. During the first 6 h, the sCD4 blocking activity was constant and equaled $K_{assoc} \approx 1.7 \times 10^9$ M^{-1}. This blocking activity agrees with unsaturated assays in Fig. 1b, verifying that the Fig. 2 assays were also unsaturated. With longer incubation, however, sCD4 blocking activity increased by more than 20-fold during a 3-h window. This rapid increase in sCD4 blocking activity at low concentrations was consistently observed with three other optimized HIV-1HXB3 stocks (33). Figure 2b demonstrates that sCD4 blocks aged viral stocks more readily than fresh viral stocks. Since HIV spontaneously sheds its envelope (42), the increase in sCD4 blocking activity with incubation suggests that some minimal number of unblocked gp120s is required for infection to occur (32). The multihit kinetics in Fig. 2a also supports this idea.

Combined effects of assay saturation and viral stock age. We further examined the combined effects of assay saturation and spontaneous viral inactivation on sCD4 blocking activity. Figures 3a and b show assay results for HIV-1HXB3 stocks established identically but then allowed to replicate virus for 2 and 6 days, respectively. After incubation (without sCD4) for 30 min at 37°C, the blocking activities (Table 2) for both assays underestimate K_{assoc}, indicating assay saturation. After 6 h, both blocking activities increased two- to threefold but their values still indicated saturation. After 13 h, the day 6 blocking activity equaled K_{assoc}, whereas the day 2 blocking activity still reflected assay saturation. These results clearly show that spontaneous viral inactivation reduces assay saturation (34, 59) and increases blocking activity.

DISCUSSION

These studies indicate that increasing target cell density decreases blocking activity (Fig. 1b). In contrast, spontaneous viral inactivation increases blocking activity (Fig. 2b). Thus, the opposing influence of these variables can completely confound measurements of blocking activity that lack proper controls. These finding have important implications for understanding blocking activity in vivo and standardizing its measurement in vitro.

The data in Table 1 (from Fig. 1b) show that infectivity assays were unsaturated and partially saturated at target cell densities of 6.25×10^4 and 1.0×10^6 ml^{-1}, respectively. Thus, somewhere between these two target cell densities, a transition in sCD4 blocking activity took place. However, a previous study of sCD4 blocking activity with HIV-1HXB3 found that assays were unsaturated over this same range of target cell densities (32). That is, blocking activity agreed with chemical measurements of K_{assoc} and no transition took place. For both studies, the method of viral stock preparation and detection of infectious events were similar. Nevertheless, the blocking activities at 1.0×10^6 target cells ml^{-1} were clearly dissimilar. These significant differences from two similar but separate studies demonstrate the need for standardizing assay conditions.

To compare blocking activities between studies or from one agent to another, quantitative infectivity assays must be unsaturated (32, 34, 59). One practical approach for meeting this requirement is to produce and store a large volume of viral stock in small aliquots. With these aliquots, a series of

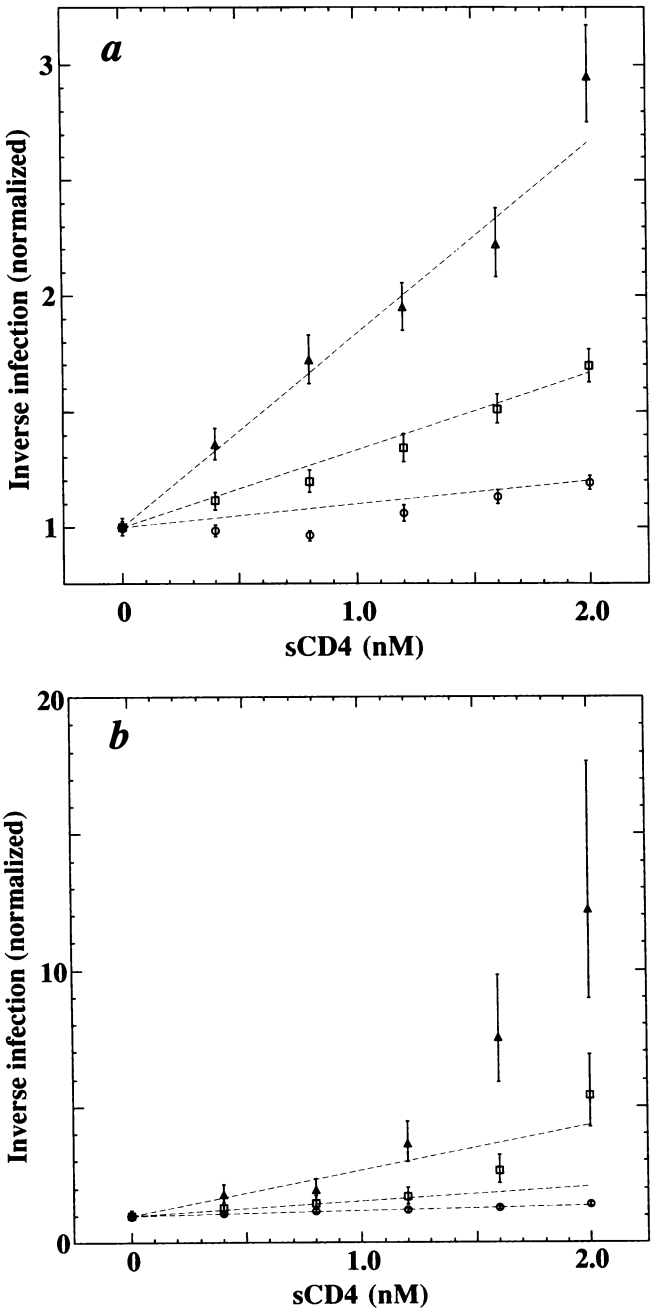


FIG. 3. Decrease in assay saturation as viral stocks spontaneously inactivate. Equivalent H9 cell cultures were inoculated with HIV-1HXB3 and harvested simultaneously after either 2 days of exponential or 6 days of chronic viral replication. The clarified stocks were then incubated at 37°C with gentle mixing. sCD4 blocking activity for day 2 (a) and day 6 (b) stocks was measured after incubation for 0.5 (○), 6 (□), and 13 (△) h, using the same assay conditions. Results are the means of eight microtiter wells (bars show ± 1 standard deviation). Dotted lines are weighted least-squares fits to the data. As HIV inactivates, sCD4 blocking increases. The blocking activity for the day 6 stock is consistently higher than for the day 2 stock (Table 2). After 13 h, the plot for the day 6 stock curves progressively upward at higher sCD4 concentrations, indicating positive synergy in blocking. This upward curvature corresponds to an increase in the molar blocking activity (defined in the legend to Fig. 1b). For the day 6 stock at 13 h, the synergy at 2.0 nM sCD4 equals 12.2/4.4 ≈ 2.8-fold. The positive synergy suggests that the day 6 stock had fewer gp120 glycoproteins per particle (on average at harvest) than did the day 2 stock.

assays at increasing target cell densities can then be performed to establish the range at which infection is proportional to cell density (Fig. 1a). As shown by the data in Table 2, sCD4 blocking activity in this range is reproducible and permits reliable measurements of K_{assoc}, even for viral stocks displaying positive synergy (Fig. 3b). Since laboratory (rapid-high) strains of HIV are easier to store and assay than field (slow-low) isolates, this approach to standardization will be considerably easier to apply to laboratory isolates. At present, however, we are unaware of any alternative method for quantitative comparisons between HIV strains.

Initial in vitro measurements of sCD4 blocking activity (10, 12, 22, 58, 61) suggested that nanomolar concentrations might have therapeutic effects in vivo. Subsequent to this, two separate sCD4-based drug trials in humans (25, 56) found that nanomolar sCD4 levels in plasma had, unexpectedly, no therapeutic effects (8). To investigate the cause of this outcome, Daar et al. (8) conducted a series of sCD4 sensitivity assays on fresh HIV-1 isolates from infected patients. Their results indicated that fresh HIV-1 isolates were 100- to 1,000-fold less sensitive to sCD4 blocking than were laboratory isolates. Although the biochemical basis for differences between field and laboratory strains was not determined, the decreased sensitivity was consistent with reductions in the chemical gp120-sCD4 K_{assoc}. Since HIV-1 and HIV-2 gp120-sCD4 K_{assoc} differ by 20-fold (32, 44), Daar et al. (8) suggested that large differences in K_{assoc} within HIV-1 might also be possible. We do not disagree with this biochemical explanation of decreased sCD4 blocking activity for fresh HIV-1 isolates. However, Fig. 1b also demonstrates that assay saturation results in significant reductions in sCD4 blocking activity. Assay saturation thus provides an alternative explanation for their observations with fresh HIV-1 isolates in vitro.

Decreasing blocking activity with increasing target cell density unifies two observations regarding therapeutic blockers and humoral immunity. First, it provides a reasonable explanation for the failure of sCD4-based therapies in vivo. Figure 1b shows that sCD4 blocking activity declines rapidly at target cell densities typical of blood, 10^6 to 10^7 ml^{-1}. Hence, sCD4 is unlikely to affect the in vivo spread of HIV in lymphoid compartments that have $CD4^+$ cell densities of 10^7 to 10^9 ml^{-1}. This suggests that therapeutic blockers (8, 25, 56) in these microenvironments need to be essentially irreversible, with K_{assoc} at least 100-fold greater than 10^9 M^{-1}. Second, this decrease also provides a disconcerting explanation for the failure of immunoglobulins to clear initial HIV infection in vivo. Preliminary investigations of 0.5β, a monoclonal immunoglobulin binding to the V3 loop in gp120 (23, 24, 54, 57), demonstrated that inverse infection plots (see Fig. 1b) were linear in concentration and sensitive to HIV-1HXB3 stock age (see Fig. 3) (33). Thus, the blocking activity of 0.5β appears to mimic that of sCD4. If other gp120-binding immunoglobulins are similar to 0.5β, this finding suggests that they will also provide little or no protection to the spread of infection in lymphoid compartments. Persistence of infection is commonly observed for viruses that infect lymphocytes and monocytes (51). The inability of gp120-binding immunoglobulins to block infection at high target cell densities therefore provides new insights into the mechanisms of such persistence.

Figures 2 and 3 show that sCD4 blocking activity increases with preincubation of viral stocks. Since spontaneous shedding of gp120 has been shown to accompany the loss of HIV infectivity (42, 45, 46), the increasing blocking activity in

both figures is presumably due to fewer gp120 knobs per active virion. Beyond this, however, the underlying explanations for these increasing activities are fundamentally different. Figure 2 shows an increasing blocking activity for unsaturated assays, whereas Fig. 3 shows it for saturated ones. For unsaturated assays, increasing sCD4 blocking activity suggests that HIV requires a critical density (or minimal number) of gp120 molecules for efficient infection of $CD4^+$ cells. This agrees with earlier observations of concentration-dependent sCD4 blocking activity, which showed enhancements after ~50% of gp120 molecules were bound (32). For saturated assays, increasing blocking activity suggests that the loss of gp120 is accompanied by a reduction in the number of chances that a virion has to infect before inactivation. This idea is consistent with the linear relationship (Fig. 1b) between sCD4 concentration and the blocking of infection in unsaturated assays.

Animal testing is a necessary step in developing an HIV vaccine. Like human transmission, experimental transmission probably takes place with HIV particles having different ages (time elapsed after budding). Consequently, immunoglobulins induced by a vaccine will have to block particles of all ages. The data in Fig. 3 suggest that newer HIV particles will be more difficult to block than older ones. Aged animal challenge stocks may thus give inappropriately high estimates of humoral efficacy in animals. In addition, the 20-fold increase in sCD4 blocking activity (from 8 to 11 h in Fig. 2b) that is accompanied by only a 2.7-fold decrease in SFU (control stock in Fig. 2a) demonstrates that increases in blocking activity are not represented by decreases in viral titer. Hence, losses in a challenge stock's titer may not accurately reflect increasing blocking activity by immunoglobulins. Consideration should therefore be given to optimizing animal challenge stocks and finding new yardsticks for evaluating such stocks. Investigations of gp120-to-p24 and gp120-to-particle ratios for assessing and monitoring challenge stock fitness in vitro are now under way.

Finally, our results indicate a need to develop rigorous screening procedures for evaluating potential HIV therapies and vaccines. The methods described in this report provide a standard and quantitative means for evaluating gp120-binding therapeutics and immunoglobulins over a variety of target cell densities that are found in vivo.

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