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High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates

(antiviral agents/affinity/AIDS/receptor/envelope)

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ABSTRACT **There is substantial evidence supporting the CD4 molecule as the principal cellular receptor for the human immunodeficiency virus type 1 (HIV-1). A number of truncated recombinant soluble CD4 (sCD4) molecules have been produced and shown to easily neutralize infection of laboratory strains of HIV-1 *in vitro*, and clinical trials using these sCD4 preparations have begun in patients with AIDS. Infectious HIV-1 titers in the plasma and peripheral blood mononuclear cells of five patients receiving sCD4 at 30 mg/day were sequentially monitored. No significant decrease in viral titers was found during therapy. Furthermore, plasma samples from eight patients with AIDS were titrated for HIV-1 with and without the addition of sCD4 *ex vivo*. Despite the addition of sCD4 at up to 1 mg/ml, there was little change in plasma viral titers. Subsequently, 10 primary HIV-1 isolates were tested for their susceptibility to neutralization *in vitro* by one preparation of sCD4. Neutralization of these clinical isolates required 200–2700 times more sCD4 than was needed to inhibit laboratory strains of HIV-1. Similar results were observed using one other monomeric sCD4 preparation and two multimeric CD4–immunoglobulin hybrid molecules. We conclude that unlike laboratory strains, primary HIV-1 isolates require high concentrations of sCD4 for neutralization. This phenomenon may pose a formidable problem for sCD4-based therapeutics in the treatment of HIV-1 infection.**

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS (1). There is strong evidence showing that the CD4 molecule on helper T cells and monocyte/macrophages is the principal receptor for HIV-1 (2, 3). Consequently, attempts have been made to create a soluble form of CD4 (sCD4) that would block HIV-1 binding and penetration. CD4 has been cloned from the T4 lymphocyte and expressed in large quantities as truncated recombinant sCD4 (4, 5). *In vitro* experiments have demonstrated significant neutralization of HIV-1 infection in T-cell lines with sCD4 at ≈100 ng/ml. These studies used HIV-1 isolates that had been propagated in the laboratory for many years. In addition, sCD4 blocked *in vitro* infection by the simian immunodeficiency viruses and the human immunodeficiency virus type 2 (HIV-2) (6), although inhibition of HIV-2 required 25 times higher concentrations of sCD4 than the tested laboratory strains of HIV-1 (7). Furthermore, several investigators have developed constructs that link sCD4 to IgG, IgM, or a cytotoxin and have shown their effectiveness *in vitro* against laboratory strains of HIV-1 [8–11].

Phase I/II clinical trials using sCD4 have been initiated based on its potent antiviral activity *in vitro* against laboratory isolates of HIV-1. We now report the lack of detectable anti-HIV-1 effect *in vivo*, as determined by quantitative viral cultures, in one phase I/II clinical trial of sCD4 despite seemingly adequate blood levels. In addition, extensive characterization of the effect of several sCD4 preparations on HIV-1 *ex vivo* and *in vitro* has subsequently revealed that much higher concentrations of sCD4 are required to neutralize unselected or minimally selected (through *in vitro* cultivation) primary viral isolates.

METHODS

Quantitation of HIV-1 *in Vivo* in the Phase I/II Clinical Trial of sCD4. The study protocol as well as the clinical and laboratory results of the phase I/II trial of sCD4 (Biogen) (5) in patients with advanced AIDS-related complex or AIDS have been described (12). In addition to measuring serum p24 core antigen levels as a marker of viral burden, an end-point-dilution culture method (13) was used for serial quantitation of HIV-1 in the peripheral blood mononuclear cells (PBMCs) and plasma of five patients treated at our center with sCD4 at 30 mg/day, intramuscularly.

Quantitation of HIV-1 in Patient Plasma After the Addition of sCD4 *ex Vivo*. Portions of cell-free plasma samples from eight AIDS patients were mixed with sCD4 (Biogen) at 0, 0.1, 1, 10, 100, and 1000 μg/ml, except in one plasma sample (patient K) where the maximum sCD4 concentration was 10 μg/ml. Subsequently, the plasma/sCD4 mixtures (at 1000, 100, 40, 10, 1, and 0.1 μl) were titrated by end-point-dilution cultures (13) to determine if there were changes in the plasma HIV-1 titers as the concentration of sCD4 increased.

HIV Isolates and Their Titrations. Primary HIV-1 isolates for *in vitro* experiments were obtained as described (13). These clinical isolates were cultured once in PBMCs from the plasma of two patients with acute HIV-1 infection (patients P and Q), an asymptomatic seropositive person (patient R), and four patients with AIDS (patients E, K, O, and S). In addition, two distinct primary viral isolates, HIV(JR-FL) and HIV(JR-CSF) (14), were kindly provided by I. Chen (UCLA School of Medicine, Los Angeles). An infectious molecular clone was obtained from a short-term culture of HIV(JR-CSF), which, when transfected into human cells, produced progeny viruses that were lymphotropic but weakly monotropic (14). In contrast, the molecularly cloned HIV(JR-FL) isolate was found to be both lymphotropic and monotropic. Finally, we isolated HIV-1AC from a patient with Kaposi sarcoma (15). This isolate was obtained from plasma after a single short-term culture using PBMCs. HIV-1AC was also propagated in H9 cells for ≈1 year. Both the early and late isolates were employed in the *in vitro* studies. Laboratory HIV isolates tested included strains HTLV-IIIB and HTLV-IIIRF and the HIV-2 isolate LAV-2_{ROD}, all of which have

Abbreviations: HIV-1 and HIV-2, human immunodeficiency virus type 1 and type 2, respectively; sCD4, soluble CD4; PBMC, peripheral blood mononuclear cell; TCID, infectious dose in tissue culture; $TCID_{50}$, median infectious dose in tissue culture; P1, passage 1; ID_{90}, 90% inhibitory dose.

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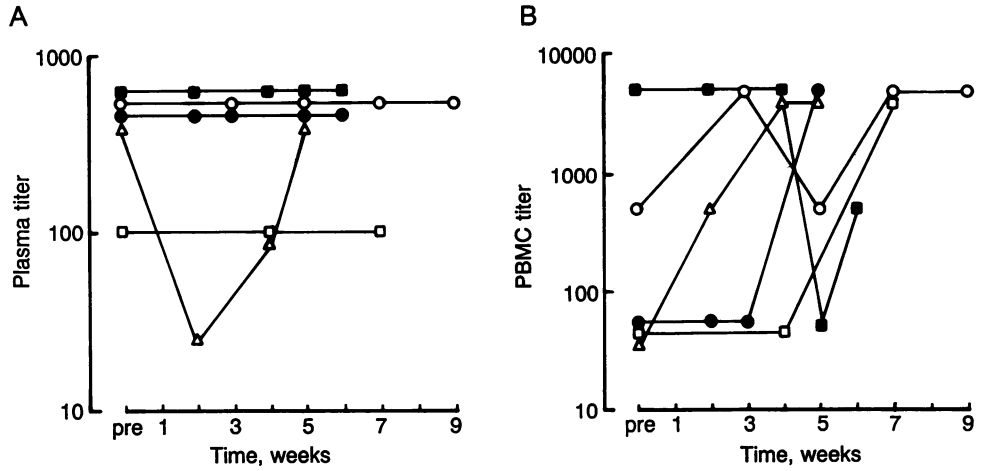

FIG. 1. Sequential HIV-1 titers in plasma (TCID per ml) (*A*) and PBMCs (TCID per 10^6 cells) (*B*) of five patients receiving sCD4. Patients: ■, A; ○, B; □, C; △, D; ●, E.

been cultured in T-cell lines for several years. These laboratory isolates were subsequently cultured and titrated in PBMCs by the end-point-dilution method prior to use in neutralization experiments *in vitro*.

Neutralization of Primary and Laboratory Isolates of HIV *in Vitro* with sCD4 or Leu3A. Neutralization assays were performed as we have described (16) using an inoculum of 50 $TCID_{50}$ (median infectious dose in tissue culture) incubated with 0, 0.1, 1, 10, 100, and 1000 μg of sCD4 (Biogen) (5) for 30 min at 37°C. On day 7, the p24 antigen levels in the sCD4-treated cultures were compared with values from an untreated culture, and results were then expressed as percent neutralization relative to the control. Similar neutralization studies were performed on a limited number of HIV-1 isolates using another monomeric sCD4 molecule (Smith Kline Beecham) (4) and the sCD4 hybrid constructs IgG–T4 and IgM–T4 provided by A. Traunecker (Bazel Institute, Switzerland) (8). IgG–T4 (90 kDa) is a hybrid molecule linking two sCD4 molecules to a portion of IgG, and IgM–T4 (700 kDa) has 10 valencies of sCD4 plus a portion of IgM. Finally, experiments to block infection by HIV-1 were also done on PBMCs and monocyte/macrophages by incubating the target cells for 30 min with Leu3A (Becton Dickinson), an anti-CD4 monoclonal antibody known to block HIV-1 infection (2, 3), prior to the addition of the viral inoculum.

RESULTS

HIV-1 Titers in Patients Receiving sCD4. End-point-dilution cultures were set up serially with the plasma and PBMCs of five patients treated with sCD4 at 30 mg/day. These patients were on therapy daily for 5–9 weeks with a mean steady-state serum sCD4 level of 156 ng/ml (12). The results of their sequential HIV-1 titers in plasma are summarized in Fig. 1*A*. Four patients had a pretherapy plasma titer of 500 infectious doses in tissue culture (TCID) per ml, and only patient D showed a transient decrease in titer to 25 TCID per ml on week 2, which returned to a level of 500 TCID per ml on week 5. HIV-1 titers in plasma samples from patients A, B, and E remained constant at 500 TCID per ml throughout the treatment period. Patient C had an initial plasma HIV-1 titer of 100 TCID per ml, which did not change during 7 weeks of treatment.

The pretherapy HIV-1 viral titers in PBMCs of these same patients ranged from 50 to 5000 TCID per 10^6 cells. Their serial PBMC titers while receiving sCD4 are summarized in Fig. 1*B*. Patient A had a pretherapy titer of 5000 TCID per 10^6 cells, which declined to 50 TCID per 10^6 cells on week 5 and subsequently increased to 500 TCID per 10^6 cells on week 6. Patients B, C, D, and E did not show decreases in HIV-1 titers in their PBMCs. In fact, patients (B, C, D, and E)

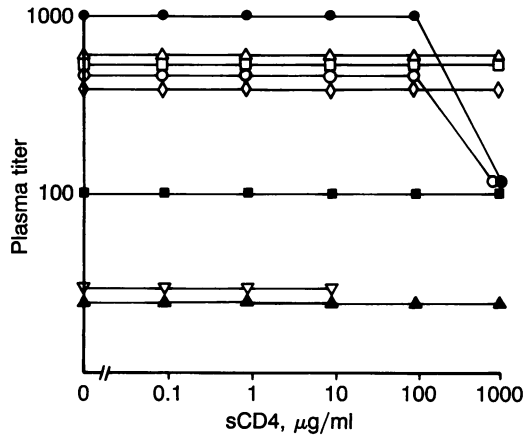

FIG. 2. HIV-1 titers in plasma (TCID per ml) after the addition of sCD4 *ex vivo*. Patients: △, A; ●, F; ○, G; □, H; ◇, I; ■, J; ▽, K; ▲, L.

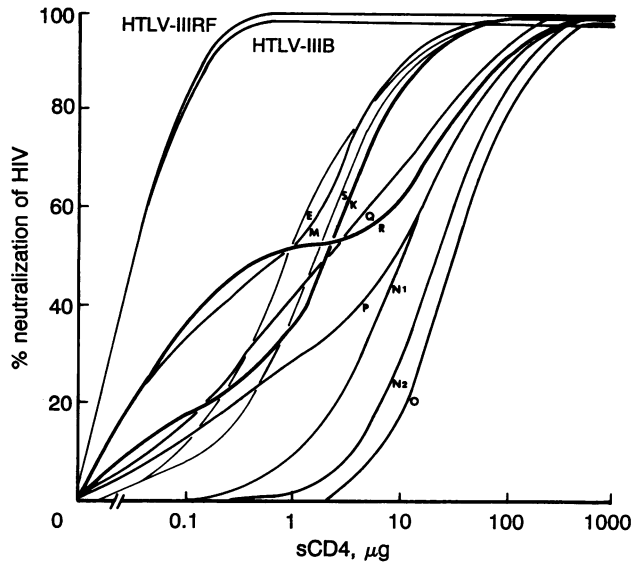

FIG. 3. HIV neutralization by sCD4 (Biogen) *in vitro*. The result for each isolate is represented by a computer-generated best-fit curve and is identified by a letter to its immediate right. Isolates: M, HIV-2 isolate $LAV\text{-}2_{ROD}$; N1, HIV(JR-FL); and N2, HIV(JR-CSF). Other primary HIV-1 isolates are identified by the letter codes used in Table 1.

Table 1. Summary of laboratory and primary HIV-1 isolates tested *in vitro*, the basic properties of each viral preparation, and the ID_{90} for four sCD4 molecules against each isolate

Patient	Isolate	Properties of viral stocks			ID_{90}, μg			
		$TCID_{50}$ per ml	p24, ng/ml	RT activity, cpm $\times 10^{-3}$/ml	sCD4(Biogen)	sCD4(S-K)	IgG–T4	IgM–T4
	HTLV-IIIB	1×10^4	300	60.5	0.07	0.03	0.02	0.11
E	E	1×10^6	286	12.3	15.0	ND	ND	0.70
K	K	2×10^3	10.0	9.30	18.0	ND	ND	ND
M	$LAV\text{-}2_{ROD}$	1×10^3	1.50	44.7	12.0	ND	ND	ND
N1	HIV(JR-FL)	2×10^4	170	98.0	110	50	2.40	0.70
N2	HIV(JR-CSF)	1×10^5	97.0	25.0	180	ND	40.0	6.00
O	O	1×10^4	1.00	613	190	ND	ND	ND
P	P	1×10^4	25.0	22.3	110	70	8.00	25.0
Q	Q	1×10^3	50.0	5.50	72.0	ND	ND	ND
R	R	1×10^3	30.0	15.5	85.0	ND	ND	ND
S	S	1×10^4	1.50	54.5	30.0	ND	ND	ND
T1*	HIV-1AC	2×10^4	573	34.7	90.0	ND	ND	17.0
T2†	HIV-1AC	1×10^3	242	320	0.90	ND	ND	1.70

S-K, Smith Kline Beecham; RT, reverse transcriptase; ND, not done.
*P1.
†After 1 year of propagation in H9 cells.

demonstrated an increase in PBMC titers during therapy. We, therefore, conclude that sCD4 (as high as 30 mg/day) did not have a detectable anti-HIV-1 effect *in vivo*, as determined by serial viral titers in plasma and PBMCs.

HIV-1 Titers in Plasma After the Addition of sCD4 *ex Vivo*. Eight patients (A, F, G, H, I, J, K, and L) with AIDS had their plasma titrated for HIV-1 with and without the addition of sCD4 *ex vivo*. Fig. 2 summarizes the quantitative results of these *ex vivo* experiments and shows that the untreated plasma titers ranged from 25 to 1000 TCID per ml. The plasma samples from patients F and G were the only specimens to demonstrate a decrease in viral titer when mixed with sCD4; however, this required a concentration of 1 mg/ml. HIV-1 titers in other plasma samples (from patients A, H, I, J, and L) were unaffected by sCD4 at 1 mg/ml, whereas the titer was unchanged in the plasma of patient K with sCD4 at 10 μg/ml. Even those specimens with low pretherapy plasma titers of 25 TCID per ml (from patients K and L) were not affected by the addition of sCD4. Overall, it appears that unselected primary HIV-1 isolates in plasma are extremely resistant to neutralization by sCD4.

Neutralization of Primary and Laboratory Isolates by sCD4 *in Vitro*. Experiments were performed to compare sCD4 neutralization of the infectivity of primary and laboratory (HTLV-IIIB, HTLV-IIIRF, and $LAV\text{-}2_{ROD}$) strains of HIV in stimulated PBMCs from normal donors. Primary viral isolates were cultured only once in PBMCs and thus referred to as passage one (P1) viruses. The results of these neutralization experiments, summarized in Fig. 3, show that, when compared to HTLV-IIIB and HTLV-IIIRF, there is a marked decrease in the effectiveness of sCD4 (Biogen) to neutralize infection by all P1 isolates as well as $LAV\text{-}2_{ROD}$. The 90% inhibitory dose (ID_{90}) of sCD4 for HTLV-IIIB and $LAV\text{-}2_{ROD}$ was 0.07 μg and 12 μg, respectively, whereas the ID_{90} for primary isolates ranged from 15 to 190 μg (Table 1). The ID_{90} for the primary isolates was 200–2700 times higher than that of HTLV-IIIB.

Fig. 4*A* shows that similar neutralization results were obtained using the sCD4 preparation (4) from Smith Kline Beecham. The ID_{90} of this sCD4 molecule for HTLV-IIIB was 0.03 μg, for HIV(JR-FL) was 50 μg, and for isolate P was 70 μg (Table 1). In addition, Fig. 4*B* shows the effectiveness of the IgG–T4 construct in neutralizing HTLV-IIIB and three P1 isolates *in vitro*. Again, the primary HIV-1 isolates were substantially more refractory to this molecule when compared with HTLV-IIIB. The ID_{90} for IgG–T4 against HTLV-IIIB was 0.02 μg, against HIV(JR-FL) was 2.4 μg, against HIV(JR-CSF) was 40.0 μg, and against isolate P was 8.0 μg (Table 1). Similar studies using IgM–T4 showed that the ID_{90} for HTLV-IIIB was 0.11 μg, compared to 0.7 μg for HIV(JR-FL), 6 μg for HIV(JR-CSF), 25 μg for isolate P, and 0.7 μg for isolate E (Table 1). Therefore, the relative resistance of primary P1 isolates to neutralization is not unique to one sCD4 preparation.

Neutralization experiments using sCD4 *in vitro* were performed using the initial P1 isolate of HIV-1AC, as well as the virus after 1 year of propagation in H9 cells. A marked difference was observed in the sensitivity of sCD4 neutralization for these two viruses (Fig. 5). The sCD4 ID_{90} for the P1 isolate was 90 μg but was only 0.9 μg for the isolate passaged in the laboratory (Table 1). It thus appears that, at least in this case, propagation of HIV-1 *in vitro* selects out viral isolates that are more susceptible to neutralization by sCD4.

Effect of Target-Cell Differences on sCD4 Neutralizing Activity *in Vitro*. Several experiments were carried out to determine the effectiveness of sCD4 in neutralizing HIV-1 infection of different target cells. The results summarized in Fig. 6*A* demonstrated that sCD4 neutralizes HTLV-IIIB and HTLV-IIIRF with equal efficiency in PBMCs and H9 cells. HIV(JR-FL) and isolate P were employed to examine the neutralizing activity of sCD4 in PBMCs and monocyte/macrophages, since both of these isolates replicate to high

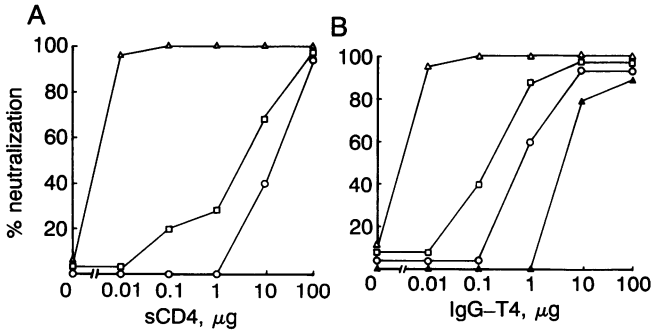

FIG. 4. Neutralization of HIV-1 by monomeric sCD4 from Smith Kline Beecham (*A*) and the multimeric IgG–T4 hybrid molecule (*B*) *in vitro*. Isolates: △, HTLV-IIIB; □, HIV (JR-FL); ▲, HIV(JR-CSF); ○, P. All isolates were 100% neutralized at 100 μg, although the data points were displayed separated one from another for the sake of clarity.

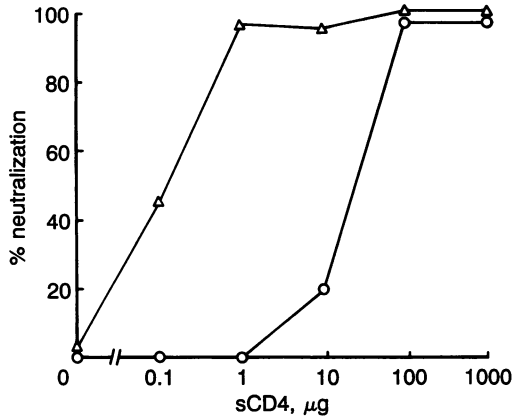

FIG. 5. Neutralization of the primary isolate HIV-1AC after a single passage in PBMCs (○) and after propagation in H9 cells for approximately 1 year (△).

levels in monocyte/macrophages. As shown in Fig. 6*B*, there was not a marked difference in sCD4 activity in these two types of target cells.

Exclusion of Two Possible Explanations for the Relative Resistance of Primary HIV-1 Isolates to sCD4. One explanation for the relative refractoriness of primary HIV-1 isolates to sCD4 was the possibility that these viruses may utilize an alternate CD4-independent mechanism to enter PBMCs. However, as shown in Fig. 6*C*, pretreatment of PBMCs with the anti-CD4 monoclonal antibody Leu3A resulted in dose-dependent inhibition of infection by HTLV-IIIB, HIV(JR-FL), and isolates P and Q. Complete inhibition was achieved with Leu3A at 1 μg/ml for these isolates. In addition, Leu3A also effectively blocked infection of monocyte/macrophages by two monotropic HIV-1 isolates [HIV(JR-FL) and P]. Therefore, the data do not support an alternate mechanism of entry for primary HIV-1 isolates.

Another explanation was the possibility that there may be excessive defective viral particles in the stock preparations of primary HIV-1 isolates. We, therefore, measured the p24 antigen concentration and the particulate reverse transcriptase activity in each virus preparation (Table 1) and then correlated these findings with the infectious titer ($TCID_{50}$ per ml) in each viral stock, as well as the ID_{90} of sCD4 against each HIV-1 isolate. Such analyses of the data outlined in Table 1 showed no evidence of excessive defective particles in the primary HIV-1 preparations (data not shown).

DISCUSSION

We have found no consistent decrease in HIV-1 titers in plasma and PBMCs of five patients receiving sCD4 (30 mg/day) for 5–9 weeks despite seemingly adequate serum levels (Fig. 1). Moreover, the addition of sCD4 (to 1 mg/ml) *ex vivo* to plasma samples from eight AIDS patients did not significantly lower their plasma HIV-1 titers, even in the two samples with relatively low viral burden (25 TCID per ml) (Fig. 2). These findings were unexpected given that previous studies have shown potent neutralizing activity of sCD4 against laboratory strains of HIV-1 *in vitro* (4–6). Therefore, several experiments were performed to address this apparent discrepancy. First, the lack of a detectable anti-HIV-1 effect of sCD4 in experiments represented by Figs. 1 and 2 could be due to an interfering factor present in plasma, such as a blocking antibody (17). The addition of heat-inactivated subneutralizing amounts of seropositive plasma to sCD4, however, did not affect its potent neutralizing activity against HTLV-IIIB (data not shown), suggesting that, at least at the amounts tested, plasma interference is not an explanation, as has been shown by others (18). This conclusion is supported by the observation that primary P1 isolates are quite resistant to sCD4 even in the absence of plasma (see below). A second possible explanation for the discrepancy is the difference in target cells given that prior studies of sCD4 against laboratory isolates of HIV-1 were performed in T-cell lines, whereas our studies were carried out in stimulated PBMCs from normal donors. The results in Fig. 6 *A* and *B*, nevertheless, did not show any significant differences in sCD4 activity using several types of target cells (H9 cells, PBMCs, and monocyte/macrophages). Lastly, the discrepancy could be due to intrinsic differences between laboratory strains and primary isolates of HIV-1. This explanation is strongly supported by the results in Figs. 3 and 5, showing that 10 primary P1 isolates were 200–2700 times more refractory to neutralization by one preparation of sCD4. Furthermore, this relative resistance of primary HIV-1 was also encountered with one other sCD4 preparation and two sCD4–immunoglobulin hybrid molecules (Fig. 4).

Although the reason for the refractoriness of primary HIV-1 isolates to sCD4 is unknown, it does not appear to be due to excessive defective interfering particles in the viral preparations. Our analyses of the data in Table 1 showed that there was no evidence of higher viral protein (p24 and reverse transcriptase) content per infectious unit in the primary HIV-1 stocks. In addition, an excess of interfering particles is unlikely to account for the relative refractoriness of

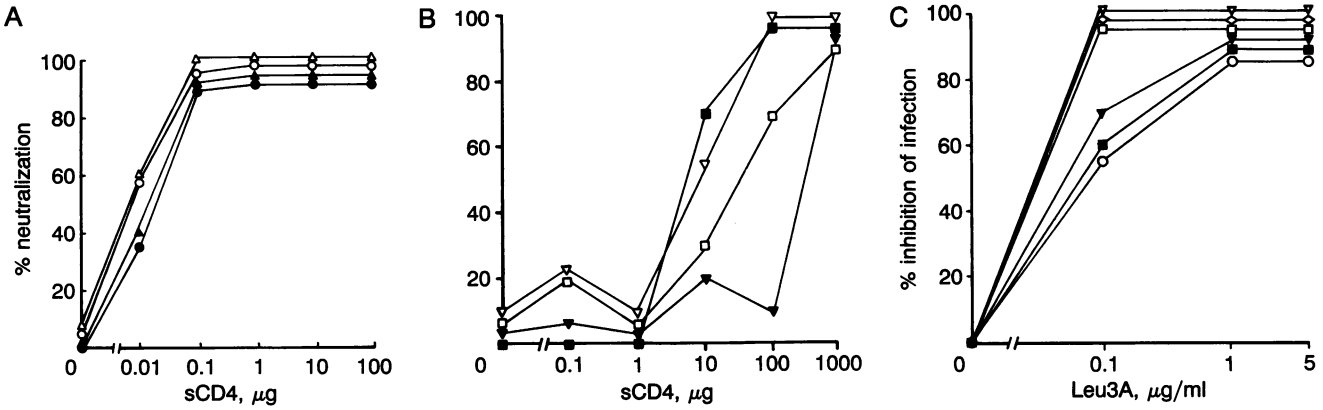

FIG. 6. (*A*) Neutralization of HIV-1 strains. ○, HTLV-IIIB in PBMCs; ●, HTLV-IIIB in H9 cells; △, HTLV-IIIRF in PBMCs; ▲, HTLV-IIIRF in H9 cells. All isolates were 100% neutralized at 0.1 μg. (*B*) Neutralization of primary isolates by sCD4 in PMBCs and monocyte/macrophages. ▽, Isolate P in PBMCs; ▼, isolate P in monocyte/macrophages; □, HIV(JR-FL) in PBMCs; ■, HIV (JR-FL) in monocyte/macrophages. (*C*) Neutralization of HIV-1 infection by pretreatment of target cells with the anti-CD4 monoclonal antibody Leu3A. ◇, Isolate Q in PBMCs; the other symbols are defined in *A* and *B*. All viruses were 100% neutralized at 1.0 μg/ml.

HIV(JR-CSF) and HIV(JR-FL) to sCD4, because these viruses are produced from the transfection of infectious molecular clones, which is a situation not prone to generate defective particles. However, the determination of the free gp120 concentration in each of the viral stocks would be needed to completely exclude interference from defective particle formation. To address another possible explanation, results in Fig. 6*C* showed that infectivity of the primary isolates was completely blocked by an anti-CD4 monoclonal antibody (Leu3A at 1 μg/ml). Therefore, the refractoriness of primary HIV-1 isolates to sCD4 is also unlikely to be due to an alternate mechanism of viral entry into PBMCs. Regardless of the explanation, however, our findings do show conclusively that primary HIV-1 isolates are rather resistant to sCD4 neutralization *in vivo*, *ex vivo*, and *in vitro*.

We speculate that the lower efficacy of sCD4 observed in our studies is due to lower binding affinities between sCD4 and gp120 of primary HIV-1 isolates. Indeed, Moore (7) has demonstrated that the relative refractoriness of HIV-2 (LAV-2_{ROD}) to sCD4 is due to a 25 times lower binding affinity between its envelope glycoprotein and sCD4 compared with that of HTLV-IIIB gp120 and sCD4 (7). By analogy, we would estimate that the gp120–sCD4 affinities for our primary HIV-1 isolates may be 100–1000 times lower than 1.5–4.0 nM, which was previously reported for the HTLV-IIIB gp120–CD4 interaction (7).

In infected persons, HIV-1 is now known to be present as a population of related yet diverse viruses termed quasispecies (19). Upon *in vitro* cultivation, selection occurs and even a minor variant can become the predominant viral form if it has a growth advantage (19). Our results in Fig. 5 show that while the quasispecies in a primary P1 HIV-1 stock were rather resistant to sCD4, a later isolate obtained after 1 year of serial passages in H9 cells was relatively sensitive to sCD4 neutralization, similar to that seen with HTLV-IIIB and HTLV-IIIRF. This observation suggests that laboratory strains of HIV-1 represent one extreme of HIV-1 populations that has been selected perhaps because of a higher gp120–CD4 affinity and thus a growth advantage in $CD4^+$ cells. Indeed, a close examination of the results in Figs. 2 and 3, showing that unselected viruses in plasma appear to be even more resistant to sCD4 than primary P1 isolates, suggests that even a single short-term passage in PBMCs *in vitro* may have already selected out viral populations that are relatively more sensitive to sCD4.

Thus the overall findings presented here have several major implications. First, the relative refractoriness of primary HIV-1 isolates to sCD4 poses a formidable problem for sCD4-based therapeutics, including those second-generation products such as sCD4–immunoglobulin hybrid constructs (8, 9) or sCD4–toxin conjugates (10, 11). We expect that much higher doses of sCD4 or its use in combination with other antiviral drugs will be necessary to achieve a consistent anti-HIV-1 effect *in vivo*.

Furthermore, it should be determined whether the relative refractoriness of primary HIV-1 isolates to sCD4 is the consequence of lower gp120–CD4 binding affinities. If this were indeed the case, then the high affinity of gp120–CD4 binding previously reported for laboratory isolates would not be representative of the envelope–receptor interaction for most HIV-1 *in vivo*.

Another implication of our findings is that although it is convenient to test anti-HIV-1 drugs using laboratory viruses and tumor cell lines, any promising agent should also be examined against unselected HIV-1 *ex vivo* and minimally selected P1 isolates *in vitro*, using normal cells as targets for infection. Despite the urgent need to develop AIDS drugs quickly, careful preclinical studies that would closely resemble the *in vivo* situation should be conducted and must not be bypassed or short changed. Similarly, basic investigations on the biology of HIV-1 should include studies on primary viral isolates that have not been highly selected under artificial conditions.

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