Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 332–336, January 1993
Medical Sciences

Soluble CD4 enhances the efficacy of immunotoxins directed against gp41 of the human immunodeficiency virus

(combination therapy/AIDS/antibody/immunoglobulin)

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Communicated by Richard M. Krause, October 7, 1992

ABSTRACT **Monoclonal antibodies specific for the gp120 or gp41 portions of the human immunodeficiency virus (HIV) envelope protein gp160 were conjugated to ricin A chain, and their immunotoxic activities against HIV-infected cells were evaluated in the presence or absence of soluble CD4 (sCD4). Immunotoxin activity was measured *in vitro* as cytotoxicity and inhibition of secretion of infectious HIV. The efficacy of anti-gp41 immunotoxins was enhanced at least 30-fold in the presence of sCD4. This effect was specific for HIV-infected cells, but not for uninfected cells, and was seen at concentrations of sCD4 as low as 0.1 μg/ml. Anti-gp120 immunotoxins were marginally inhibited at higher concentrations of sCD4. Flow cytometry analyses showed that sCD4 increased the expression of gp41 on the surface of infected cells and increased internalization of gp120 and gp41. These data suggest that sCD4 alters the cellular trafficking of HIV envelope proteins. These findings also have important implications for the therapeutic use of anti-HIV immunotoxins and may be generalizable to other immunotoxins as well.**

CD4 is an integral membrane glycoprotein of helper T lymphocytes and serves as the cellular receptor for human immunodeficiency virus (HIV) (1). CD4 is bound by the extracellular portion of the viral envelope protein gp120. Upon binding to CD4 the envelope protein undergoes conformational changes, including release of free gp120 from virions (2, 3), loss of the envelope spikes as viewed by electron microscopy (4), and increased exposure of epitopes on the NH_2 terminus of the transmembrane portion of the envelope protein gp41 (5). These conformational changes are probably the earliest steps in the infectious process following the binding of HIV to its cellular receptor. They may also represent potential opportunities for therapeutic intervention.

One approach to the treatment of HIV infection is the elimination of cells that actively produce the virus. To this end, agents have been developed that use either antibodies or CD4 to deliver a toxic moiety to cells expressing the HIV envelope at the cell surface (6–11). These must bind and be internalized to kill the target cell. Accordingly, these reagents are not only potential therapeutic agents but also probes that may be used to study the cellular trafficking of retroviral proteins.

We have produced a panel of immunotoxins that consists of anti-envelope antibodies directed against different epitopes on gp120 and gp41 that are coupled to ricin A chain (RAC) (7). Because the addition of soluble CD4 (sCD4) has been shown to alter the conformation of the HIV envelope protein, the effect of sCD4 on the efficacy of anti-gp160 immunotoxins was tested. It is demonstrated that the *in vitro* efficacy of anti-gp41 immunotoxins was enhanced by at least 30-fold in the presence of sCD4. This effect is most likely due to an increase in the expression of gp41 epitopes on the surface of target cells as well as to increased rates of internalization of gp41.

MATERIALS AND METHODS

Antibodies, Immunotoxins, and sCD4. Specificities of monoclonal antibodies used in this study are listed in Table 1. Antibodies 41.1 and 41.4 bind to the immunodominant region of gp41, ≈60 amino acids toward the COOH terminus from the fusion domain. Antibodies were coupled to RAC at a molecular ratio of 1:1 to produce immunotoxins as described elsewhere (7). CD4-PE40 (9, 11) was obtained from Upjohn, as was $sCD4_{183}$, which consists of the NH_2-terminal two domains of CD4 (16).

Cellular Assays. The following cell lines were used in these studies: H9 $CD4^+$ T-cell leukemia H9 (provided by M. Reitz, National Cancer Institute, Bethesda, MD); H9 cells persistently infected with the infectious molecular clone of HIV, NL4-3 (17, 18); and H9 cells infected with the HIV isolate $HTLV\text{-}III_{MN}$ (AIDS Research and Reference Reagent Program, Rockville, MD). Virtually 100% of H9/NL4-3 cells were actively producing HIV, whereas <1% of H9/MN secreted virus. $CD4^+$ HeLa cell line 1022 was used to detect infectious HIV (19).

Immunotoxin efficacy was monitored by two previously described assays (7). The cytotoxic effect of immunotoxins was measured on H9/NL4-3 cells in a 72-hr assay. Cells (3×10^4) were plated in 0.2 ml of RPMI 1640 medium with 10% fetal calf serum in flat-bottom 7-mm microtiter wells (Flow Laboratories). Protein synthesis was measured during the final 16 hr of culture as [^{35}S]methionine (0.5 μCi per well; 1 Ci = 37 GBq) incorporation, and the results are reported as counts per minute (cpm). The ability of the immunotoxin to inhibit the production of infectious HIV was determined using a specific focal immunoassay (FIA) (7, 19, 20). Monolayers of $CD4^+$ HeLa cells were plated at 3×10^4 cells per 2-ml (16-mm) well. HIV-infected cells were treated for 24–48 hr with the immunotoxin and/or sCD4. The cells were then plated in serial dilutions (3×10^5 to 100 cells per well, the range depending upon the particular cells being tested) on the monolayer of $CD4^+$ HeLa cells. Twenty-four hours later the cells were washed off of the monolayer. Foci of HIV-infected cells were detected by immunoperoxidase staining 2 days later. The results are reported as the percent of input cells that scored as infectious centers.

Flow cytometry was performed on H9/NL4-3 cells on a Becton Dickinson Facstar using the indicated monoclonal antibody and a species-specific fluorescein-conjugated anti-immunoglobulin secondary antibody as described elsewhere (7). Antibodies were used at a concentration of 10 μg/ml,

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Abbreviations: HIV, human immunodeficiency virus; FIA, focal immunoassay; RAC, ricin A chain; sCD4, soluble CD4.

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Table 1. Monoclonal antibodies used in this study

mAb	Species	Specificity	Reactivity with HIV isolates	Source or ref.
T7	Mouse	Irrelevant antigen	None	12
924	Mouse	gp120, V3 loop amino acids 313–324	IIIB only	7
F58	Mouse	gp120, V3 loop amino acids 309–317	Broadly reactive	13
10B	Humanized*	Irrelevant antigen	None	14
41.1	Human	gp41, amino acids 579–604	Broadly reactive	J.M.
41.4	Human	gp41, amino acids 579–604†	Broadly reactive	J.M.

Specificity of monoclonal antibodies (mAbs) was determined on synthetic peptides; amino acid numbering conforms to HXB-2 envelope sequence (15).
*Antibody 10B is a chimeric antibody with mouse variable regions and human constant regions.
†Antibody 41.4 reacts weakly with a peptide representing these amino acids, binds to recombinant gp160 with higher affinity than 41.1, and cross-competes with 41.1. Thus 41.4 most likely binds to a conformational epitope in this region.

determined to be a saturating concentration by prior titration. Cells were incubated sequentially with each antibody for 1–2 hr at room temperature in the presence of 0.1% sodium azide. To determine rates of internalization of HIV antigens, monoclonal antibodies were directly conjugated with 6-(fluorescein-5-carboxamido)hexanoic acid, succinimydal ester (Molecular Probes) according to the manufacturer's protocol at a fluorescein-to-protein ratio of 7:1. Cells (10^6) were incubated with sCD4 (1 μg/ml) for 2 hr at 37°C and then placed in the presence of the directly fluoresceinated antibody (30 μg/ml) at 37°C in RPMI 1640 medium containing 10% fetal calf serum. At the indicated time they were transferred to cold medium containing 0.5% sodium azide. Fluoresceinated antibody detecting cell surface antigen was quenched by incubation of the cells for 1 hr at 4°C in anti-fluorescein antibody (Molecular Probes) at a concentration previously determined to be in 3-fold excess for inhibiting cell surface fluorescence. Total fluorescence (unquenched) and internal fluorescence (quenched) were determined by flow cytometry. Calculations were based upon the mean fluorescent value for the entire cell population studied (i.e., no fluorescence gates were set). Internal fluorescence at each time point was calculated by subtracting the quenched fluorescence at time zero (i.e., the background fluorescence before any internalization could occur) from the quenched fluorescence at the indicated time.

RESULTS AND DISCUSSION

Because the addition of sCD4 has been shown to alter the conformation of the HIV envelope protein, the effect of sCD4 on the efficacy of anti-gp160 immunotoxins was examined. To determine if there was enhancement of immunotoxin efficacy, immunotoxins were tested at different concentrations in the presence or absence of 1 μg of sCD4 per ml (Fig. 1). Immunotoxin efficacy was measured as inhibition of protein synthesis in persistently infected H9/NL4-3 cells. Two different immunotoxins were tested: 41.1-RAC and 41.4-RAC. CD4-PE40 was included as a control. In the absence of sCD4, CD4-PE40 and 41.4-RAC were equally efficacious, whereas 41.1-RAC gave equivalent inhibitions at a third of the dose of the others. When sCD4 was added to CD4-PE40, the expected inhibition of its action was observed. The activities of 41.4-RAC and 41.1-RAC were significantly enhanced by sCD4. Equivalent toxicity by 41.4-RAC was seen at 0.01 μg/ml in the presence of sCD4 and 0.3 mg/ml in its absence, indicating a 30-fold enhancement of immunotoxin efficacy. With 41.1-RAC, complete suppression of protein synthesis was seen in the presence of sCD4 at the lowest dose tested (0.01 μg/ml), indicating that the enhancement was at least 30-fold, possibly greater. A similar dose–response was performed on uninfected H9 cells at immunotoxin concentrations as high as 100 μg/ml (data not shown). There was no suppression of protein synthesis in uninfected cells by either 41.1 or 41.4-RAC in the presence or absence of sCD4 at any immunotoxin concentration. In contrast, nonspecific toxicity of CD4-PE40 on uninfected H9 cells was seen at concentrations >30 μg/ml.

The concentration of sCD4 required to produce an effect on immunotoxins was next determined (Fig. 2). Anti-gp41 immunotoxins (41.1 and 41.4-RAC) were tested at the suboptimal concentration of 0.08 μg/ml. Maximal enhancement of their effect could be observed at concentrations of sCD4 as low as 0.1 μg/ml. sCD4 has been reported to produce the release of free gp120 from HIV virions and infected cells and to decrease the exposure of gp120 antigens on the surface of infected cells (3, 5). We therefore tested anti-gp120 immunotoxins F58 and 924-RAC at effective doses (2 μg/ml) and looked for diminution of their effect by sCD4. At concentrations of sCD4 >1 μg/ml, a modest decrease in the efficacy of the 924, but not F58, immunotoxin was noted (Fig. 2).

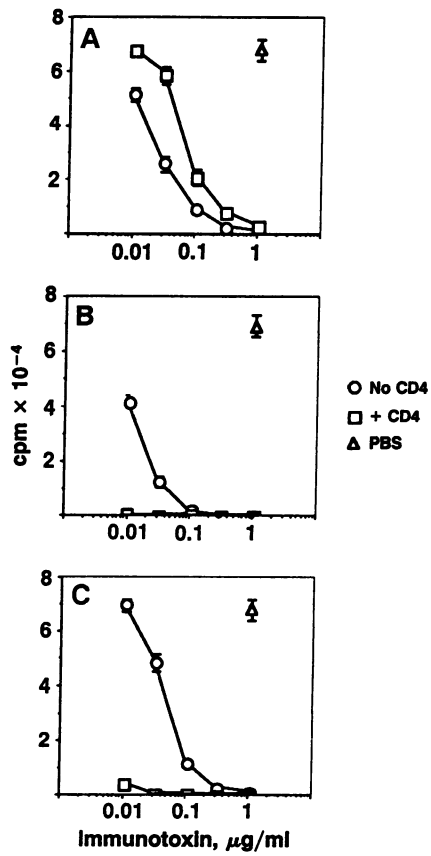

FIG. 1. Dose–response of immunotoxins in the presence or absence of sCD4. The ability of three different immunotoxins [CD4-PE40 (A), 41.1-RAC (B), 41.4-RAC (C)] to inhibit protein synthesis in H9/NL4-3 cells was determined in the presence or absence of sCD4 (1 μg/ml). Immunotoxins were tested over a 100-fold range of concentrations. PBS indicates protein synthesis in the absence of any immunotoxin.

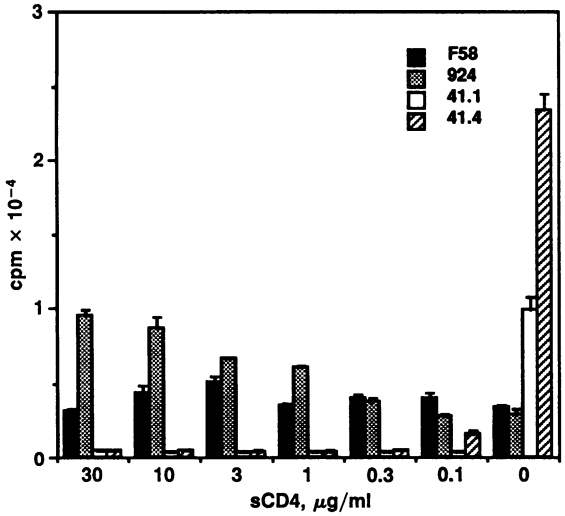

FIG. 2. Dose–response of sCD4 effect upon immunotoxins. The effect of anti-gp41 and anti-gp120 immunotoxins was measured on H9/NL4-3 cells in the presence of various concentrations of sCD4. Anti-gp41 immunotoxins 41.1-RAC and 41.4-RAC were tested at a suboptimal concentration (0.08 μg/ml), whereas anti-gp120 immunotoxins 924-RAC and F58-RAC were tested at an efficacious dose (2 μg/ml). [^{35}S]Methionine incorporation in H9/NL4-3 cells in the absence of immunotoxin was 84,889 cpm.

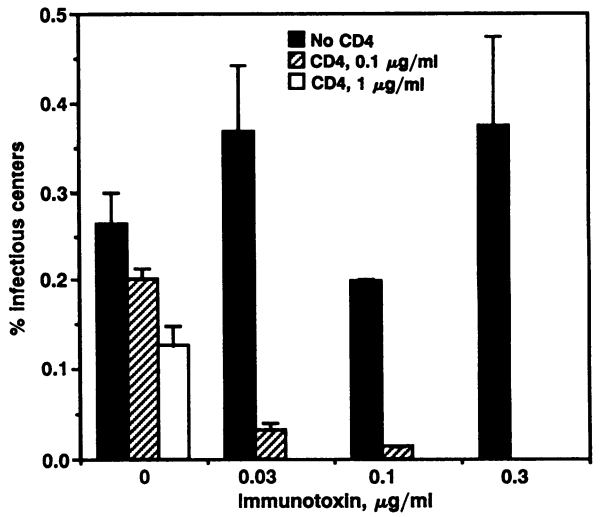

FIG. 3. Inhibition of production of infectious HIV by combinations of immunotoxin and sCD4. H9 cells infected with the MN strain of HIV were incubated for 48 hr in the presence of the indicated concentrations of sCD4 and 41.4-RAC. Serial dilutions of the cells (3 × 10^5 to 10^3 cells per well) were then transferred to monolayers of CD4$^+$ HeLa cell line 1022. Three days later, the number of foci were enumerated by immunoperoxidase.

The nature of the conformational change induced by sCD4 has not been precisely defined. To probe this, monoclonal antibodies directed against regions of gp160 that are exposed on the soluble envelope protein, but not functionally accessible on cell-associated gp160, were used, hoping that sCD4-induced conformational changes may expose these cryptic epitopes on the cell surface. The following monoclonal antibodies (with their epitopes indicated) were coupled to RAC: H11 (amino acids 472–477), C6 (amino acids 91–99), B13 (amino acids 256–264), 110.1 (amino acids 200–217), C-1 (amino acids 492–498), and C8 (amino acids 727–732). All but the last recognize epitopes in gp120. When tested in the presence or absence of sCD4 these immunotoxins had no effect on H9/NL4-3 cells (data not shown). Thus, sCD4-induced conformational changes do not include the exposure of these epitopes on the cell surface.

To demonstrate that the effect of sCD4 on immunotoxin action was not restricted to H9/NL4-3 cells, a different HIV isolate and different cell types were tested. The FIA was used to assess the efficacy because in these situations only a small proportion of the cells was expressing viral antigens, rendering the measurement of cytotoxicity on these populations meaningless. The combined effect of 41.4-RAC and sCD4 on H9 cells infected with the HIV isolate MN is shown in Fig. 3. The immunotoxin alone was ineffective at all three doses tested. sCD4 alone showed a small, but significant, dose-related neutralization of the infection of the indicator CD4$^+$ HeLa cells by the MN virus. However, when immunotoxin and sCD4 were used in conjunction, virtually complete inhibition of HIV infectivity was seen at all doses tested. A similar experiment was performed using CEM and phytohemagglutinin (PHA)-stimulated blast cells acutely infected with NL4-3 strain. With the CEM cells, the addition of sCD4 enhanced the efficacy of the 41.4 immunotoxin at least 10-fold (testing was not done for greater increases). Additive efficacy of sCD4 and 41.4-RAC was also seen on the PHA blasts (not shown).

The mechanism of sCD4 enhancement was studied using flow cytometry. In the first experiment, indirect immunofluorescence was used to assess the increase in cell surface antigen expression following treatment of H9/NL4-3 cells with sCD4 (10 μg/ml) at 37°C (Fig. 4*A*). The data indicated that there was an increase in exposure of gp41 epitopes and a decrease in gp120. This effect was seen at lower doses of sCD4 as well and was consistent with previously published work (5). However, additional factors may also account for the enhancement of immunotoxin efficacy. Since internalization of immunotoxins is important for their function, the effect of sCD4 on internalization of antibodies bound to envelope antigens was examined. In this experiment directly fluoresceinated antibodies were incubated with H9/NL4-3 cells at 37°C for increasing periods of time. Cell metabolism was then paralyzed with sodium azide. The fluorescence of antibody bound to cell surface antigens was quenched with anti-fluorescein antibody. Internalized antibody was then detected by flow cytometry (Fig. 4*B*). sCD4 (1 μg/ml) enhanced the internalization of antibodies 924 (anti-gp120) and 41.4 (anti-gp41) but not the irrelevant control antibody 10B. This effect was not due to increased binding of the antibody to sCD4-treated cells, since it was seen with antibody 924, whose binding is decreased by sCD4.

These data indicate that in addition to the previously described effects of sCD4 on the conformation of the envelope protein, sCD4 also affects the intracellular circulation of the HIV envelope protein(s) in infected cells. Increased internalization, taken together with the alterations in cell surface antigen exposure, can explain the effects of sCD4 on anti-gp41 and anti-gp120 immunotoxins. Enhancement of anti-gp41 immunotoxin activity is a result of increased exposure of gp41 epitopes on the cell surface and increased internalization. sCD4 has little effect on the efficacy of anti-gp120 immunotoxins because decreased antigen exposure is offset by increased rates of internalization.

Under the experimental conditions used here, there was a synergistic interaction between sCD4 and anti-gp41 immunotoxins. The efficacy of these immunotoxins was enhanced at least 30-fold by the addition of sCD4. There was no indication that the enhancement was accompanied by any increase in nonspecific toxicity. CD4-PE40 also acts by binding to HIV envelope protein on the surface of infected cells, is internalized, and then kills the cells. The data shown here demonstrate that CD4-PE40 and the anti-gp41 immunotoxins 41.1-RAC and 41.4-RAC have very similar *in vitro*

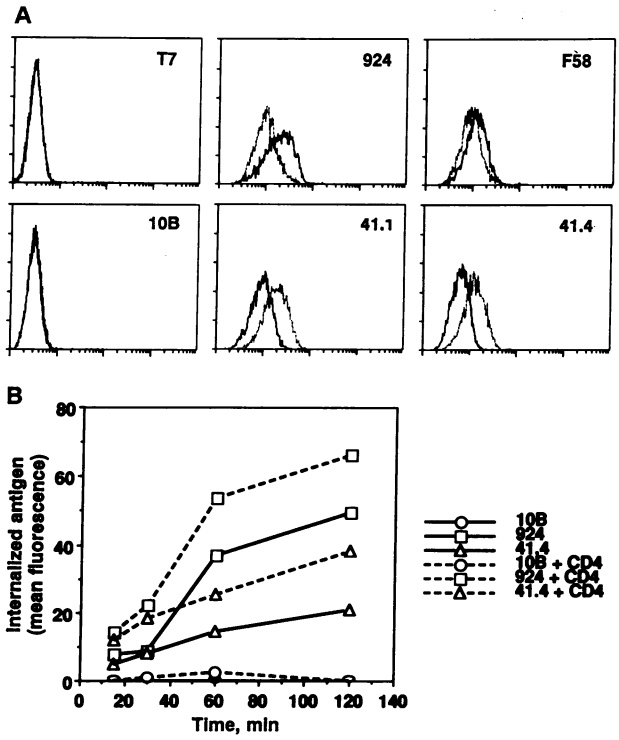

FIG. 4. Flow cytometric analyses of sCD4 effects on H9/NL4-3 cells. (*A*) Indirect immunofluorescent analyses of antigen exposure following incubation in the presence (broken line) or absence (solid line) of sCD4 (10 μg/ml) for 2 hr at 37°C are shown. Antibodies T7 and 10B are irrelevant control antibodies, 924 and F58 are anti-gp120, and 41.1 and 41.4 are anti-gp41. Cell number is shown on the ordinate; log fluorescence intensity is shown on the abscissa. (*B*) The effect of sCD4 on the internalization of envelope antigens was measured by flow cytometry. Cells were incubated with directly fluoresceinated antibodies for the indicated time at 37°C and then poisoned with sodium azide. Cell surface fluorescence was quenched with an anti-fluorescein antibody. Internalized antigen was then detected by flow cytometry. Changes in cell surface antigen expression (mean fluorescence) induced by sCD4 were a decrease of 46 fluorescence units for antibody 924 and an increase of 111 for antibody 41.4 (at the 30-min time point).

dose–response curves on persistently infected cells in the absence of sCD4; but when sCD4 is added the RAC-conjugated monoclonal antibodies are considerably more potent (Fig. 1). Further advantages of monoclonal antibody-based immunotoxins include a longer serum half-life (21, 22) and less nonspecific toxicity on uninfected tissue culture cells (see above). However, it should be noted that CD4-PE40 may be somewhat more active against acutely infected cells than the anti-gp41 immunotoxins (S.H.P., unpublished), most likely due to the additive effect of neutralization and toxin activity that we have previously described (7). If immunotoxins are shown to have clinical utility, it is likely that treatment will involve panels of different immunotoxins because of immunogenicity and HIV variation (23). There will be a place for immunotoxins based on monoclonal antibodies and CD4 toxins in the treatment of AIDS.

We have shown *in vitro* enhancement of immunotoxin action by sCD4 but at this time can only speculate about whether this effect will be true *in vivo* as well. It is clear that the concentrations of immunotoxin and sCD4 at which we observe *in vitro* effects are readily attainable *in vivo* (24–26). Moreover, with the introduction of CD4-immunoglobulin derivatives, the serum half-lives of CD4 and immunotoxins are almost identical (21, 22, 24). Thus, it should be possible to coadminister the immunotoxin and CD4-immunoglobulin *in vivo* and obtain the effects we have reported here.

The exact nature of the conformational change induced by the binding of CD4 to the gp120/gp41 complex is still a matter of conjecture (5, 27, 28). There are likely multiple points of attachment between CD4 and gp120 (28). Following this binding, gp120 dissociates from gp41 to a variable degree (2, 27). There is a concomitant increase in the exposure of the NH_2 terminus of gp41 (5), which contains the fusion domain. This in turn allows the next step in viral entry, membrane fusion, to proceed. The increase in exposure of the NH_2 terminus of gp41 may result from any or all of the following: (*i*) conformational changes in the gp120/gp41 complex, (*ii*) changes in the state of gp41 oligomerization, (*iii*) dissociation of gp120 from gp41 (5). Two pieces of data reported here bear on these issues. The first is the failure of sCD4 to cause exposure of cryptic epitopes on either gp120 or gp41, suggesting that there are not global conformational changes induced in envelope protein by CD4 binding. Second, we demonstrated that CD4 binding enhances the internalization of gp120 and gp41. Similar findings are observed when cell surface receptors bind their ligands and are internalized via coated pits, a phenomenon associated with increased oligomerization of the receptor. By analogy, our data suggest that CD4 binding may alter the state of gp120/gp41 oligomerization.

A caveat on the utility of the results described here is that patient-derived HIV may be less sensitive to sCD4-mediated neutralization than are laboratory strains (27). It may also be true that patient-derived isolates are less susceptible to sCD4-mediated enhancement of anti-gp41 immunotoxin action and, for that matter, to the toxic effects of CD4-PE40.

The enhancement of immunotoxin activity described here may be generally applicable to other immunotoxins. gp160/sCD4 may be considered analogous to other receptor/ligand systems. A number of immunotoxins directed against cellular receptors for growth factors have been proposed for the therapy of cancer. Examples include transferrin, epidermal growth factor, platelet-derived growth factor, and interleukin 2. Increased internalization of many receptors in the presence of their ligands has been demonstrated. Thus the internalization of anti-receptor immunotoxins, and consequently their therapeutic efficacy, may be enhanced by the addition of free ligand. Such an observation would greatly increase the utility of immunotoxins in the therapy of cancer as well as AIDS.

We thank C. Horgan, B. Wahren, G. Lewis, and B. Chesebro for the antibodies used in these studies; G. Tarpley and D. Batts for sCD4 and CD4-PE40; J. Stahl for helpful discussions regarding the study of internalized antigens; G. Spangrude and D. Brooks for assistance with the flow cytometer; Susan Smaus and Carole Smaus for secretarial assistance; and John Swanson, Bruce Chesebro, Britta Wahren, and Kim Hasenkrug for careful review of the manuscript.

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