

# CD4-*Pseudomonas* Exotoxin Conjugates Delay but Do Not Fully Inhibit Human Immunodeficiency Virus Replication in Lymphocytes In Vitro

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## Abstract

The CD4 molecule is a high affinity receptor for the human immunodeficiency virus (HIV) envelope glycoprotein (gp160 or gp120). This glycoprotein is expressed on the surface membrane of cells infected with HIV. It has, therefore, been suggested that a soluble form of CD4 might be used as a targeting agent to deliver toxins selectively to cells infected with HIV. We demonstrate that CD4-*Pseudomonas* exotoxin A (PE) conjugates inhibit the proliferation of gp160-transfected Chinese hamster ovary cells and block HIV replication in virus-infected H9 cells. However, this inhibition of HIV replication appears to be incomplete since virus replication occurs following removal of the toxin conjugates from these cultures. Moreover, CD4-PE conjugates delay but do not inhibit HIV replication in human peripheral blood lymphocytes. These studies suggest that such conjugates should be assessed only as potential adjunctive therapies in the acquired immunodeficiency syndrome. (*J. Clin. Invest.* 1990. 86:1684–1689.) Key words: acquired immunodeficiency syndrome therapy • antiviral drug • HIV • *Pseudomonas* exotoxin conjugates

## Introduction

The CD4 molecule is a high affinity receptor for the human immunodeficiency virus (HIV) envelope glycoprotein (1). Anti-CD4 monoclonal antibodies block HIV infection of target cells in vitro (2, 3). Cells not expressing the CD4 molecule may become susceptible to HIV infection following transfection with the CD4 gene (4). Moreover, it has recently been shown that a soluble form of the extracellular portion of the CD4 molecule created through recombinant DNA technology can block HIV infection of cells in vitro (5–9).

It has been suggested that a soluble form of CD4 may prove valuable in the therapy of HIV-infected individuals by directly competing with cell surface expressed CD4 molecules for the binding of virus. In fact, recent studies in simian immunodeficiency virus-infected rhesus monkeys and HIV-infected humans suggest that this approach may be of therapeutic value (10–12). It has also been proposed that soluble CD4 might be employed for the targeting of toxins to cells infected with HIV. Thus, cells infected with HIV and expressing virus envelope glycoprotein on their surface might specifically bind a CD4-

toxin conjugate and be killed as a result of that interaction. In vitro experiments have indicated that this approach may be feasible (13–16).

We have recently initiated studies to explore the possible use of CD4-toxin conjugates in the treatment of AIDS. In these experiments, we demonstrate that CD4-*Pseudomonas* exotoxin (PE)<sup>1</sup> conjugates can inhibit HIV replication in cell populations in vitro. However, this inhibition of virus replication appears to be incomplete.

## Methods

**Materials.** Murine anti-CD4 (19Thy5D7) and anti-CD8 (7PT3F9) monoclonal antibodies (MAbs) were provided by Dr. S. F. Schlossman (Dana Farber Cancer Institute, Boston, MA). Mitomycin-C was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human IL-2 was provided by Hoffman-LaRoche (Nutley, NJ). The Chinese hamster ovary (CHO) cell lines expressing HIV gp160/120 and mullerian inhibiting substance were gifts of Dr. M. Rosa (Biogen, Inc., Cambridge, MA).

**CD4-toxin proteins.** The CD4-PE constructs used in these studies were expressed by *Escherichia coli* renatured and purified by immune-affinity chromatography using an anti-CD4 monoclonal antibody bound to BrCN-activated Sepharose as described by Winkler et al. (manuscript in preparation) (Fig. 1). Both contained amino acids 1–181 of the human CD4 molecule, its two amino-terminal Ig-like domains (16a, 16b). The shorter construct, CD4-PE(364), contained amino acids 250–613 of PE, its 364 carboxy-terminal amino acids (16c). This consists of domains II (the portion required for translocation of the toxin into the cytoplasm of a target cell), Ib, and III (the portion responsible for ADP-ribosylation of elongation factor 2) (16d). The longer construct, CD4-PE(392), contained, in addition, the carboxy-terminal 28 amino acids of domain I which are involved in cell recognition (16d).

**Cell proliferation experiments.** CHO cells transfected with genes encoding HIV envelope glycoprotein gp160 or mullerian inhibiting substance were plated in 96-well plates at a density of  $2 \times 10^4$  cells/well in MEM alpha medium (without ribonucleotides and deoxyribonucleotides) (Gibco Laboratories, Grand Island, NY) supplemented with 10% dialyzed fetal bovine serum (Gibco) and 4 mM L-glutamine. After an overnight incubation, medium was removed and replaced by fresh medium containing CD4-PE. For blocking experiments, 0.5  $\mu$ M recombinant soluble CD4 was also added. After 24 h incubation, 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA) was added and incubation was continued for another 24 h. Cells were harvested and lysed on a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA) and incorporated radioactivity was determined by scintillation counting.

**Virus.** H9 cells infected with HIV-1 (HTLV-III B strain) were used as a source of virus. The cell line was maintained in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FCS (Flow Laboratories, Inc., McLean, VA), L-glutamine (2 mM), penicillin (50 U/ml), and gentamycin (50  $\mu$ g/ml).

1. **Abbreviations used in this paper:** CHO, Chinese hamster ovary; MMC, mitomycin C; PE, *Pseudomonas* exotoxin A; RT, reverse transcriptase.

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Received for publication 25 January 1990 and in revised form 4 June 1990.

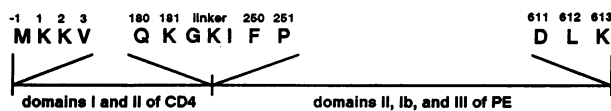
*J. Clin. Invest.*

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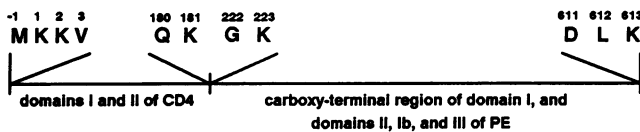
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Volume 86, November 1990, 1684–1689

### CD4(181)-PE(364)



### CD4(181)-PE(392)



**Figure 1.** Structure of CD4-PE recombinant proteins utilized in these studies. Both hybrid toxins used in these experiments contained amino acids 1–181 of the human CD4 molecule, its two amino-terminal domains. The shorter of the two constructs contained residues 250–613 of PE, the 364 carboxy-terminal amino acids; the longer contained an additional 28 amino acids comprising the carboxy-terminal region of domain I, the cell recognition domain of PE.

**Cultures of HIV-infected H9 cells with CD4-PE.**  $2 \times 10^4$  HIV-1-infected H9 cells were placed in 24-well culture plates (Gibco Laboratories). Increasing concentrations of CD4-PE and then  $2 \times 10^5$  uninfected H9 cells were introduced into each well. Cells were washed after 3 d in culture; new medium and CD4-PE were then added to each well. CD4-PE was added to each well again on day 5. Thereafter, medium was changed and new CD4-PE was added to each well every 2 d. Cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

**Peripheral blood lymphocyte (PBL) preparation.** Heparinized blood was obtained from HIV-1 seropositive or seronegative volunteers. PBLs were isolated from the heparinized blood by Ficoll-diatrizoate density gradient centrifugation. They were washed with PBS, resuspended in RPMI 1640 supplemented with 10% FCS at a concentration of  $1 \times 10^6$ /ml, and activated with 10 µg/ml concanavalin A (Con A) for 3–4 d. CD8+ cells were depleted from these Con A-activated PBLs by the panning method (17, 18). PBLs were incubated at a concentration of  $1.5 \times 10^7$ /ml for 40 min at 4°C with anti-CD8 MAb (7PT3F9) in ascites form at a dilution of 1:125. The cells were then washed twice with PBS and resuspended in PBS at a concentration of  $4 \times 10^6$  cells/ml. 3 ml of this cell suspension was plated on a 10-cm plastic petri dish coated with 10 µg rabbit anti-mouse Ig (Dako Corp., Santa Barbara, CA) and preincubated with 10 ml of PBS supplemented with 2% FCS. Cells were incubated on these dishes for 70 min at 4°C. The dishes were then swirled and cells in the supernatant were harvested. These cells were used as a CD8+ cell-depleted population. CD8+ cell-depleted PBLs were maintained in RPMI 1640 medium supplemented with 10% FCS and 20 U/ml IL-2 at a concentration of  $1 \times 10^6$  cells/ml.

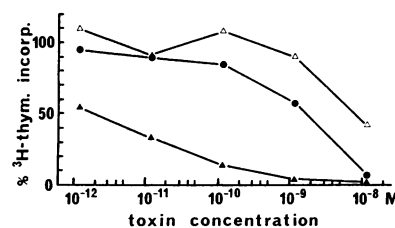
**Reverse transcriptase (RT) assay.** RT activities of culture supernatants were measured as described (19). Briefly, 1.4 ml of each supernatant was centrifuged in a 1.5-ml Eppendorf tube at 12,000 g for 90 min. The supernatant was removed and pelleted virus was incubated on ice for 10 min with 20 µl of dissociation buffer (0.01 M Tris-HCl, pH 7.3/0.2% Triton X-100/0.001 M EDTA/0.05 M dithiothreitol/0.06 M KCl). 15 µl of dissociated virus solution was mixed with 60 µl of assay mixture (0.05 M Tris-HCl, pH 8.3/0.007 M MgCl<sub>2</sub>/0.06 M KCl/0.08 mg of poly(rC)-oligo(dG) primer per ml/0.007 M dithiothreitol/3.3 µCi of  $\alpha$ -[<sup>32</sup>P]dGTP [3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL]) and incubated at 37°C for 60 min. 60 µl of each sample was dropped onto a Whatman 3 disk. Each disk was washed in a beaker with 5% trichloroacetic acid/2% sodium pyrophosphate, rinsed, dried, and radioactivity of each disk was measured.

## Results

**CD4-PE inhibits replication of CHO cells expressing HIV gp160.** The toxicity of a CD4-PE hybrid toxin was first assessed on CHO cells expressing the HIV envelope glycoprotein gp160. CHO cells transfected with either gp160 or a control gene coding for mullerian inhibiting substance were maintained in culture for 48 h with increasing concentrations of CD4-PE(364). [<sup>3</sup>H]thymidine incorporation by these cells was then measured as an indication of cell replication. As shown in Fig. 2, a 50% inhibition of [<sup>3</sup>H]thymidine incorporation by gp160-transfected CHO cells was seen at a  $1.5 \times 10^{-12}$  M concentration of CD4-PE. This inhibition of replication was dependent upon the gp160 interaction with CD4-PE, since it was completely eliminated by the addition of soluble CD4 at a concentration of 0.5 µM. No significant CD4-PE toxicity on control CHO cells expressing mullerian inhibiting substance protein was seen at concentrations less than  $2.0 \times 10^{-9}$  M. Thus, a significant “window” exists between the specific toxicity of CD4-PE for gp160-expressing CHO cells and the nonspecific toxicity of this conjugate for CHO cells expressing an irrelevant molecule. However, that toxicity window appeared to be smaller for H9 than for CHO cells, since 50-nM CD4-PE exhibited significant toxicity for uninfected H9 cells (data not shown).

**CD4-PE inhibits HIV replication in vitro.** The ability of two different CD4-PE constructs to block HIV replication in vitro was then assessed. Uninfected H9 cells, a HIV-permissive cell population, were cultured with H9 cells that were chronically infected with HIV. Increasing concentrations of two different CD4-PE constructs were maintained in these cultures and in vitro HIV replication was monitored by quantitating RT activity in the culture supernatants. RT activity was detected in these supernatants by day 7 of culture in the absence of CD4-toxins. However, both CD4-toxin constructs completely inhibited HIV replication when maintained in culture at a 6.0-nM concentration (Fig. 3). CD4-PE(392) appeared to block HIV replication marginally more effectively than CD4-PE(364) at a 0.2-nM concentration. CD4-PE(392) was also marginally more efficient than CD4-PE(364) in lysing gp160-transfected CHO cells (Winkler et al., manuscript in preparation). The CD4-PE constructs were not cytotoxic for uninfected H9 cells at those concentrations (data not shown).

**HIV replication occurs after removal of CD4-PE from cultures.** We then sought to determine whether the introduction of CD4-PE in vitro for a finite period of time permanently eliminated replicating HIV in these cultures. Uninfected H9



**Figure 2.** CD4-PE inhibits replication of CHO cells expressing the HIV gp160. gp160-transfected CHO cells (▲) or CHO cells transfected with mullerian inhibiting substance (△) were cultured in the

presence of CD4-PE(364) for 48 h. Soluble CD4 at a 0.5-µM concentration was also added to such a culture of CD4-PE-treated cells (●). [<sup>3</sup>H]thymidine was added for the last 24 h of culture, cells were harvested, and incorporated radioactivity was determined. This was then expressed as a percentage relative to untreated cells.

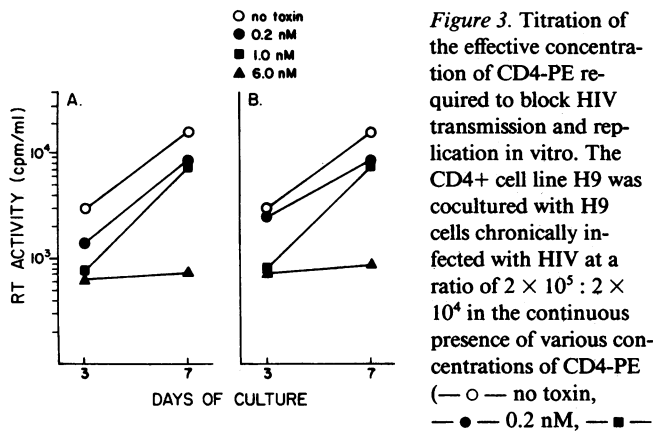


Figure 3. Titration of the effective concentration of CD4-PE required to block HIV transmission and replication in vitro. The CD4+ cell line H9 was cocultured with H9 cells chronically infected with HIV at a ratio of  $2 \times 10^5 : 2 \times 10^4$  in the continuous presence of various concentrations of CD4-PE (—○— no toxin, —●— 0.2 nM, —■— 1.0 nM, —▲— 6.0 nM). RT activity in culture supernatants was measured as an indication of HIV replication. A and B indicate the results of studies done using CD4-PE(392) and CD4-PE(364), respectively.

cells and HIV-infected H9 cells were cocultivated in the presence of 12.0-nM CD4-PE(392). CD4-PE was, however, removed from the cultures on day 3, day 7, or day 10. In each of these instances, HIV replication was detected immediately after CD4-PE was removed from the cultures (Fig. 4). Therefore, a 12.0-nM concentration of CD4-PE inhibited HIV replication only as long as it was present in culture. Moreover, when CD4-PE was maintained in a similar culture system for 10 d at a 6.0-nM rather than 12.0-nM concentration, a concentration that fully abrogated HIV replication for a 7-d culture period, virus replication was readily demonstrable by day 10 (Fig. 5).

CD4-PE does not block transmission of HIV from infected to uninfected H9 cells. Thus, CD4-PE exposure appeared to delay but did not abort the generation of RT activity in these cultures. This HIV replication could reflect the transmission in culture of HIV from virus-infected to virus-free H9 cells. It could, however, also simply represent the gradual expansion of the HIV-infected H9 cells originally introduced in vitro as a source of virus. To differentiate between these two possibilities, cell-associated HIV was introduced into the cultures of uninfected H9 cells in a cell population which could not expand and support HIV replication. We have previously found that mitomycin C (MMC)-treated HIV-infected H9 cells can

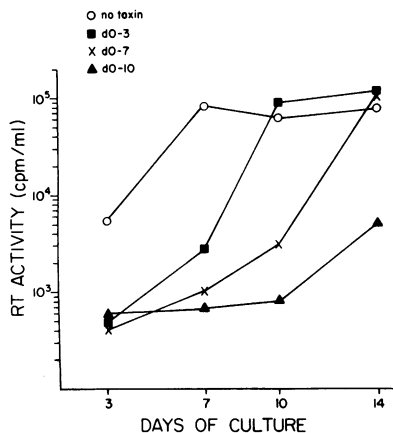


Figure 4. The inhibition of HIV replication in H9 cells persists only as long as 12.0-nM CD4-PE is present in culture.  $2 \times 10^5$  H9 cells were cocultured with  $2 \times 10^4$  HIV-infected H9 cells in the continuous presence of 12-nM CD4-PE(392) for the period of time indicated. —○—, no toxin; —■—, day 0-3; —×—, day 0-7; —▲—, day 0-10.

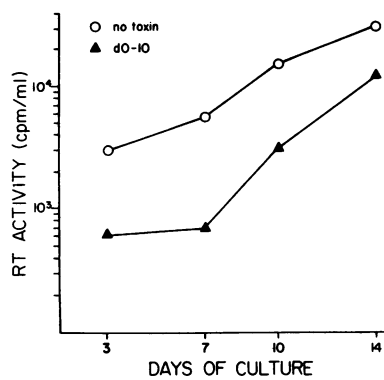


Figure 5. HIV replication in H9 cells is delayed but not fully abrogated in the presence of 6.0-nM CD4-PE.  $2 \times 10^5$  H9 cells were cocultured with  $2 \times 10^4$  HIV-infected H9 cells in the continuous presence of 6-nM CD4-PE(392) from day 0 to day 10 of culture. —○—, no toxin; —▲—, 6 nM CD4-PE.

serve as a source of cell-associated HIV in culture while not supporting the continued replication of this virus (data not shown). Therefore, MMC-treated, HIV-infected H9 cells were placed in culture with uninfected H9 cells in the presence of 6.0-nM CD4-PE. While no RT activity was detected in culture supernatants of the MMC-treated, HIV-infected H9 cells alone, RT activity was generated when uninfected H9 cells were introduced in vitro (Fig. 6). Therefore, HIV transmission from infected to uninfected H9 cells occurred in culture in the continuous presence of CD4-PE.

CD4-PE delays but does not block HIV infection of PBLs. We next assessed the ability of CD4-PE to block HIV infection in a more biologically relevant cell population, PBLs. Con A-activated, CD8+ cell-depleted PBLs obtained from an uninfected donor were cocultured with MMC-treated, HIV-infected H9 cells. CD4-PE was maintained at different concentrations in the cultures from day 0 to day 7. Virus replication was detected as early as day 3 in the control culture, with peak RT activity noted on day 7 of culture (Fig. 7). CD4-PE delayed HIV infection of PBLs in a dose-dependent manner. CD4-PE at a 0.2-nM concentration did not inhibit HIV replication (data not shown). The peak RT activity generated in a culture maintained in 1.0-nM CD4-PE was a bit lower than that of the control culture (data not shown). Finally, no significant increment in inhibitory activity was observed in cultures main-

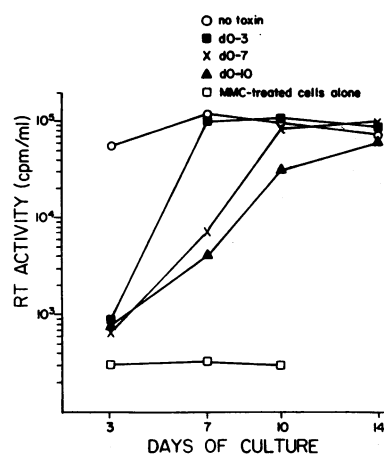
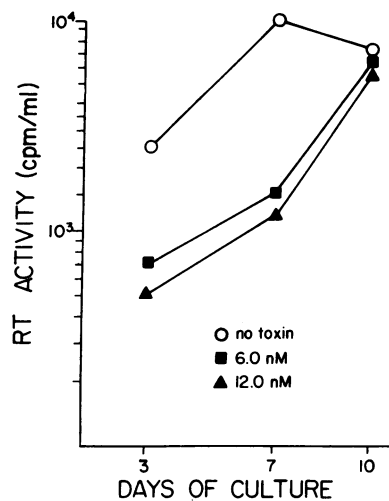


Figure 6. CD4-PE blocks transmission of infection from mitomycin C-treated HIV-infected H9 cells to uninfected H9 cells, albeit transiently.  $2 \times 10^5$  H9 cells were cocultured with  $2 \times 10^4$  mitomycin C-treated, HIV-infected H9 cells in the presence or absence of 6-nM CD4-PE (392) for various periods of time. As a negative control,  $2 \times 10^4$  mitomycin C-treated, HIV-infected cells were also maintained alone in culture. —○—, no toxin; —■—, day 0-3; —×—, day 0-7; —▲—, day 0-10; —□—, mitomycin C-treated, HIV-infected H9 cells alone.



**Figure 7.** CD4-PE blocks HIV infection of PBLs with HIV, however, only transiently.  $1 \times 10^6$  CD8+ cell-depleted, Con A-activated PBLs were cocultured with  $2 \times 10^4$  mitomycin C-treated HIV-infected H9 cells in the presence of 6.0 nM or 12.0 nM CD4-PE(392) from day 0 to day 7. —○—, no toxin; —■—, 6.0 nM CD4-PE; —▲—, 12.0 nM CD4-PE.

tained in 12.0 nM as compared to 6.0-nM CD4-PE. The onset of HIV replication was clearly delayed in the cultures treated with 6.0 nM and 12.0-nM CD4-PE, with RT activity only one tenth that of the control cultures on day 7. However, by day 10 of culture, only 3 d after removal of CD4-PE, RT activity in supernatants of the CD4-PE-treated cell populations was equal to that of the control cells.

*CD4-PE does not inhibit transmission of HIV from infected to uninfected PBLs.* Thus, CD4-PE appears to inhibit HIV replication in PBLs, but only transiently. Such a transient inhibition could, however, considerably delay HIV expansion in cell populations susceptible to infection. To assess this possibility, the efficacy of CD4-PE was assessed in another in vitro system. The system chosen for this evaluation was one that should closely approximate the conditions under which a CD4-toxin must work in order to be effective in vivo in the HIV-infected patient. PBLs from an HIV-infected individual were placed in culture with Con A for 4 d. CD8+ cells were then eliminated from these activated PBLs. A similarly prepared CD8+ cell-depleted, Con A-activated PBL population from an uninfected individual was added to these cells and CD4-PE was introduced into these cultured cells. This system, therefore, allowed us to assess the ability of CD4-PE to block transmission of HIV from the rare virus-infected circulating lymphocyte of the individual to other potentially susceptible lymphocytes. Although HIV transmission in this culture system was fully inhibited by an anti-CD4 MAb, neither an inhibition nor a delay in HIV replication was achieved using CD4-PE (Fig. 8).

## Discussion

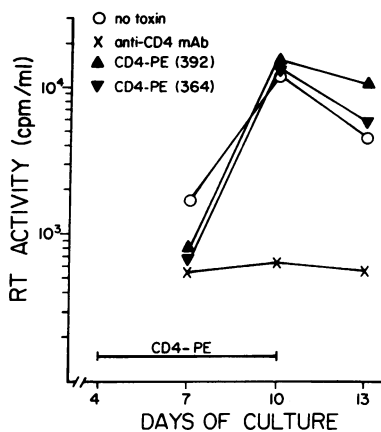
These studies demonstrate that CD4-PE conjugates inhibit the replication of gp160-transfected CHO cells and block replication in virus-infected H9 cells at nanomolar concentrations. However, this inhibition of virus replication does not appear to be complete since virus replication occurs following removal of the toxin conjugates from these cultures. Moreover, CD4-PE conjugates delay but do not inhibit HIV replication in human PBLs.

The CD4-PE conjugates used in these studies appear to be as potent in their ability to inhibit HIV replication in vitro as

those utilized by other investigators. Till et al. demonstrated greater than 90% inhibition of HIV replication in H9 cells in the presence of 0.1-nM CD4-ricin (14). Berger et al. demonstrated such an inhibition at a 1-nM concentration, with complete inhibition at a 10-nM concentration of CD4-PE (15). In the present studies, the ID<sub>50</sub> of the CD4-PE constructs for gp160-transfected CHO cells was 0.0012 nM and complete inhibition of cell replication was observed at a 1.2-nM concentration of CD4-PE. Maximal inhibition of HIV replication in H9 cells was seen using these conjugates at a 6-nM concentration.

In these studies, we explored the use of CD4-PE conjugates in in vitro systems which should be indicative of the value of such conjugates in a therapeutic setting. In studies of monoclonal antibodies coupled to toxins, it has become apparent that delivery of such conjugates to individuals over a prolonged period of time can result in significant toxin-induced nonspecific tissue injury. Moreover, it is also clear that repeated dosing of such conjugates eventually results in the generation of toxin-specific antibody responses that can neutralize the therapeutic efficacy of these molecules (20). For these reasons it is assumed that toxin conjugates can be used at most intermittently in therapy (20). We therefore assessed viral replication in vitro before and after removing CD4-PE from cultures of HIV-infected H9 cells. Our observation that viral replication can be demonstrated soon after removal of the CD4-PE-toxin conjugates from these cultured HIV-infected cells is, therefore, troubling.

In further attempts to study the CD4-PE conjugates in physiologically relevant systems, we also assessed the ability of these conjugates to inhibit viral spread from a limited number of HIV-infected PBLs to uninfected PBLs. We were unable to detect CD4-PE inhibition of virus spread from infected to uninfected cells. A number of explanations might be proposed to account for the apparent inefficiency of CD4-PE to inhibit HIV spread and replication under these in vitro conditions.



**Figure 8.** CD4-PE does not block transmission of HIV from infected to uninfected cells in PBLs of an HIV-infected individual. PBLs obtained from an HIV-infected individual were activated with Con A for 4 d. CD8+ cells were then removed by a panning procedure.  $1 \times 10^6$  similarly treated CD8+ cell-depleted PBLs derived from a healthy individual were cocultured with  $1 \times 10^5$  of

the cells from the HIV-infected individual in the presence of 6.0-nM CD4-PE(392), 6.0-nM CD4-PE(364), or a 1:400 dilution of monoclonal anti-CD4 antibody ascites (19Thy5D7), an antibody that blocks binding of HIV to CD4. The CD4-PE or antibody were present in the culture from day 4 to day 10. Coculture with cells of the normal individual began on day 4 of culture. —○—, no toxin; —×—, anti-CD4 mAb (19Thy5D7); —▲—, CD4-PE(392); —▼—, CD4-PE(364).

HIV may spread from cell to cell in lymphocyte populations before envelope glycoprotein is expressed on the surface of these cells in quantities sufficient to make the cells optimal targets for CD4-toxin conjugates. If this, in fact, occurs, the use of the CD4 toxins to inhibit spread of virus in PBLs may be of limited value. CD4 expression is also considerably greater on PBLs than on H9 cells. PBLs may, therefore, be more susceptible to infection by limited numbers of HIV particles than are H9 cells. If this were true, CD4-PE might be less efficient at blocking HIV infection in PBLs than in H9 cells.

The experiments described in these studies do not contradict findings reported by other investigators working with CD4-PE. Chaudhary et al. did not measure HIV replication in CD4-PE-treated cells. They only assessed the proliferative capacity of CV-1 cells infected with vaccinia-gp160 and HIV-infected A3.01 cells after a brief exposure to CD4-PE (13). Till et al. similarly only measured the proliferative activity of CD4-PE-treated HIV-infected H9 cells (14). Although Berger et al. did demonstrate a CD4-PE-induced inhibition of RT generation by HIV-infected cells *in vitro*, they studied virus replication only in A3.01 cells (15). These experiments assessed CD4-PE-mediated inhibition of HIV replication in H9 cells and, more importantly, in human PBLs. Moreover, in the experiments most central to these studies, CD4-PE was assessed for its ability to block transmission of HIV from infected to uninfected PBLs. The kinetics of HIV replication in H9 cells and lectin-activated human PBLs, and the susceptibility of these virus-infected cells to killing by CD4-PE, might be expected to differ substantially from those of HIV-infected A3.01 cells.

While these studies suggest that CD4-PE may not completely eliminate replicating virus from an infected cell population, CD4-toxin conjugates may still prove of value in the therapeutic armamentarium against AIDS. CD4 conjugates prepared with cell toxins more potent than PE may be more efficient at eliminating virus-infected cells than CD4-PE. CD4-toxin conjugates may also prove more toxic *in vivo* than *in vitro*. Those issues notwithstanding, the data in the present study clearly demonstrate that CD4-PE does delay the spread of HIV in culture. It is, therefore, possible that such a conjugate may prove a useful adjunct to other modalities of treatment in AIDS.

## Acknowledgments

We thank M. Hirsch for providing blood samples from HIV-1 infected patients, and D. Brosseau for preparing this manuscript.

This work was supported by National Institutes of Health grants AI-20729, CA-50139, and RR-00168, and funds from Biogen, Inc. Dr. Letvin is a recipient of an American Cancer Society Faculty Research Award.

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