Antiviral Research, 16 (1991) 267 279

ADONIS 016635429100374R

AVR 00509

Effects of a soluble CD4 and CD4-*Pseudomonas* exotoxin A chimeric protein on human peripheral blood lymphocytes: lymphocyte activation and anti-HIV activity in vitro

Kathleen L. Rubino, W. Gary Tarpley and Judith A. Nicholas

Cancer and Infectious Diseases Research, Upjohn Laboratories, Kalamazoo, Michigan, U.S.A.

(Received 30 October 1990; revision accepted 19 April 1991)

Summary

Recombinant sCD4-based proteins were evaluated for their effects on antigen-stimulated proliferation of human peripheral blood mononuclear cells (PBMC) and for antiviral activity against PBMC infected with human immunodeficiency virus (HIV_{D34}). Two sCD4-based proteins were solubilized, refolded, and purified to homogeneity from recombinant *E. coli* and consisted of the 178 amino-terminal residues of CD4 fused with the translocating and catalytic domains of *Pseudomonas* exotoxin A (sCD4-PE40) or 183 amino-terminal residues of CD4 (sCD4-183); a third sCD4 consisting of 369 amino acids of CD4 was purified from recombinant mammalian cells for comparative purposes (sCD4-369). Increasing molar concentrations of these sCD4s were evaluated for inhibition of PBMC proliferation induced by alloantigen (MLR), by tetanus toxoid (TTOX), or in response to crosslinking with antibody to CD3 (OKT3). In addition, the concentrations of each protein required to inhibit replication of the HIV_{D34} isolate in primary PBMC was determined by quantitation of HIV p24 antigen released into supernatant fluids by infected cells. By comparing antiviral activity with anti-proliferative activity a relative estimate of the selectivity index for each recombinant sCD4 was determined. Proliferation of PBMC in response to alloantigen or OKT3 was less sensitive to inhibition than proliferation induced by TTOX, and the selectivity indices estimated for sCD4-PE40 were 170, 170 and 17, respectively. The selectivity index for sCD4-

Correspondence to: K.L. Rubino, Cancer and Infectious Diseases Research, Upjohn Laboratories, 301 Henrietta St., Kalamazoo, MI 49007, U.S.A.

183 was greater than 350 under all assay conditions. Comparative evaluation of alloantigen-stimulated proliferation with antiviral activity of sCD4-183 versus sCD4-369 suggested that the *E. coli*-derived sCD4-183 may have a higher selectivity index under these conditions than its mammalian cell-derived counterpart.

Soluble CD4; sCD4-183; sCD4-PE40; AIDS; HIV; *Pseudomonas* exotoxin A

Introduction

Human immunodeficiency virus (HIV) infects lymphocytes through specific binding of the viral envelope glycoprotein, gp120, with CD4 molecules expressed by the helper subset of T lymphocytes and a limited number of other types of cells (reviewed by Farrar et al., 1988). Soluble forms of CD4 (sCD4) containing various extracellular domains of the protein have been obtained from recombinant systems and are potent inhibitors of HIV infection in vitro (Fisher et al., 1988; Hussey et al., 1988; Deen et al., 1988; Traunecker et al., 1988; Smith et al., 1987; Clapham et al., 1989). Because of the strong anti-HIV activity of sCD4 in vitro, trials in non-human primates and phase I/II clinical trials have been initiated (Schooley et al., 1990; Kahn et al., 1990; Watanabe et al., 1989). Although additional data are required to provide an assessment of the value of receptor-based therapy for acquired immunodeficiency syndrome (AIDS), these initial results have led to a search for additional sCD4-based therapeutics which may have selective advantages.

The gp120-binding activity of CD4 resides within its amino-terminal variable-region-like domain (Richardson et al., 1988; Berger et al., 1988; Traunecker et al., 1988), thus facilitating expression in *E. coli* of a truncated form of sCD4 (sCD4-183) capable of binding gp120 in solution (McQuade et al., 1989) and having potent antiviral activity (Garlick et al., 1990). The expression in *E. coli* of a chimeric protein containing the amino-terminal 178 residues of CD4 fused with the translocating and ADP-ribosylating domains of *Pseudomonas* exotoxin A (sCD4-PE40) has also been described (Chaudhary et al., 1988). sCD4-PE40 also binds gp120 in solution (Chaudhary et al., 1988; McQuade et al., 1989) and shows selective cytotoxicity for a variety of cell lines infected with HIV-1 or expressing gp120 (Chaudhary et al., 1988; Berger et al., 1990).

The normal physiological role of CD4 is as a co-receptor for antigen recognition and signal transduction in the helper subset of T lymphocytes (reviewed by Janeway, 1988). The T-cell receptor for antigen recognizes foreign peptides presented at the surface of antigen-presenting cells in the context of polymorphic regions of class II molecules, encoded by the major histocompatibility complex (MHC). This recognition occurs in conjunction with CD4 which interacts with nonpolymorphic regions of class II MHC molecules. The co-

association of CD4 and the T-cell receptor with MHC facilitates signal transduction and T-cell activation which culminate in cellular proliferation. Since all of the sCD4-based therapeutics described to date possess determinants identical to those within the natural CD4 ligand, and because these therapies may be required for extended treatment periods owing to the chronic nature of HIV infection, it is important to determine whether the concentrations of sCD4s required for inhibition of HIV may also interfere with normal immune functions involving MHC class II-associated recognition of antigen. Toward this goal, we have evaluated anti-proliferative effects of sCD4-PE40 and sCD4-183 in 3 different antigen-stimulation assays in comparison with their respective anti-HIV_{D34} activities as an in vitro estimate of their respective selectivity indices. These data may be useful for predicting safe and efficacious dosing strategies for the clinical management of HIV infection.

Materials and Methods

Cells, medium, and monoclonal antibodies (mAb)

PBMC were obtained from healthy, non-risk-group adult volunteers by leukapheresis or by venipuncture and purified by centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ). PBMC were used immediately without freezing for virus infectivity studies. For proliferation assays, PBMC were first frozen in liquid nitrogen at 2 to 4 $\times$ 10^7/ml in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) containing 20% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 10% dimethylsulfoxide (DMSO; Fisher Scientific, Itasca, IL) to provide a uniform source of cells for all proliferation assays and for storage of large PBMC batches from multiple donors. Assays were performed in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine (Whittaker), 10 mM Hepes buffer (Biologos, Inc, Naperville, IL), 50 μM β-mercaptoethanol (BioRad, Richmond, CA) and 50 μg/ml gentamicin sulfate (Whittaker). B-lymphocyte cell lines (B-LCL) were established by published procedures (Issekutz et al., 1982). Briefly, PBMC were transformed with Epstein-Barr virus containing supernatant fluids from the B95-8 marmoset cell line (American Type Culture Collection, ATCC, Rockville, MD) and cultured in the presence of cyclosporin A (Sandoz Pharmaceuticals, E. Hannover, NJ) in the described medium. Monoclonal antibodies used to characterize proliferation assays were OKT4a (anti-CD4; Ortho Diagnostics, Raritan, NJ), MT151 (anti-CD4; Boerhinger Mannheim, Indianapolis, IN), Leu3a (anti-CD4; Becton Dickinson, Mountain View, CA), L243 (anti-HLA-DR; Becton Dickinson), and Leu6 (anti-thymocyte CD1 control; Becton Dickinson). OKT3 was collected from hybridoma culture (Clone OKT3; ATCC), purified by chromatography on protein A sepharose, and provided as a generous gift of Dr. Ann Berger.

PBMC proliferation assays

All proliferation assays were performed in a volume of 200 μl medium; assay conditions were empirically determined to yield highest specific proliferative activity. For the mixed lymphocyte reaction (MLR), B-LCL were treated with 25 μg/ml mitomycin C (Sigma, St. Louis, MO) for 15 min at 37°C, washed, and 0.5 to 1 × 10^4 allogeneic B-LCL per well were used to stimulate proliferation of 2 × 10^5 responding PBMC. Controls of B-LCL + medium or PBMC + medium were included in each MLR to ensure that mitomycin C treatment abrogated any B-LCL proliferation and to confirm that our assay was, in fact, a 'one-way MLR' with only the PBMC responding to the allogeneic B-LCL. MLR assays were incubated 4 days, pulse-labeled overnight with 1 μCi/well [^{3}H]thymidine (Amersham, Arlington Heights, IL) and harvested onto filter discs using a Brandell cell harvester (Gaithersburg, MD). Mean incorporation of radiolabeled precursor into DNA was quantitated from triplicate samples within each assay using a Beckman LS 5200 spectrometer. For TTOX assays, 4 × 10^5 PBMC were cultured with 0.735 U/ml TTOX (Michigan Dept. Public Health, Lansing, MI) for 7 days at 37°C. PBMC were pulse-labeled with 1 μCi [^{3}H]thymidine for the final 4 h, then harvested and quantitated as described above. For OKT3-induced proliferation, 2 × 10^5 PBMC were stimulated with 12.5 ng/ml OKT3 antibody for 36 h, then pulse-labeled with 1 μCi [^{3}H]thymidine overnight before harvest and quantitation of proliferation as described above. Incorporation of [^{3}H]thymidine by PBMC in the absence of antigen (allogeneic B-LCL, TTOX or OKT3) was negligible.

sCD4-PE40, sCD4-183 and sCD4-369

sCD4-PE40 and sCD4-183 were expressed at high levels in *E. coli* from pBR322-based vectors as previously described (Hussey et al., 1988; Garlick et al., 1990). sCD4s were solubilized from inclusion bodies and purified to apparent homogeneity (>95%) by a combination of immunoaffinity and size exclusion chromatographies. Purified proteins were shown to be homogenous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining with silver, by N-terminal sequencing, and by amino acid analysis as described in detail (Garlick et al., 1990). sCD4-369 was purified from the medium of recombinant mammalian cells engineered to secrete sCD4-369. Composition and purity were extensively analyzed by methodology as described above (SDS-PAGE, N-terminal sequencing and amino acid analysis) which indicated sCD4-369 preparations to have purity equivalent to the material derived from *E. coli* (i.e. >95%). As an additional control, a chimeric protein consisting of human transferrin fused with the translocating and catalytic domains of *Pseudomonas* exotoxin A (Tr-PE40) was purified from recombinant *E. coli* and was provided as a generous gift of Dr. Ira Pastan (unpublished). Tr-PE40 is toxic for cells with transferrin receptors, known to be expressed in abundance at the surface of proliferating human lymphocytes.

HIV infectivity experiments

The inhibitory effects of sCD4-PE40, sCD4-183 or sCD4-369 on HIV replication in human PBMC were determined as previously described (Garlick et al., 1990) by the DIAGEN Corporation, Dusseldorf, F.R.G. Briefly, PHA-stimulated primary cultures of unfrozen PBMC were infected in triplicate with the laboratory-adapted D34 isolate of HIV-1, similar to HTLV-III_B, (Rubsamen-Waigmann et al., 1986; von-Briesen et al., 1987) and the appropriate sCD4 was added immediately after virus adsorption. Three and 4 days after infection, the levels of HIV p24 synthesized in PBMC and released into the medium were quantitated by enzyme-linked immunosorbent assay using a monoclonal capture antibody to HIV p24 and recombinant p24 calibration standard (HIV p24 Core Profile ELISA; DuPont, Wilmington, DE). To ensure that the experiments were performed at nontoxic concentrations of sCD4-PE40, sCD4-183 or sCD4-369, the viability of drug-treated and untreated PHA-stimulated blasts was determined by cell counting in parallel experiments (data not shown).

Results

Effect of sCD4-PE40 on PBMC proliferative responses

In previous studies we characterized the PBMC response to alloantigen in the MLR as dependent upon CD4 and MHC class II interaction and demonstrated that 17 nM sCD4-PE40 had no effect on the MLR response of a single PBMC donor (Berger et al., 1990). To determine if this lack of effect was restricted to the response of a single donor or more broadly applicable, we investigated the effect of sCD4-PE40 on the MLR responses of 13 different PBMC donors (Fig. 1A). Tr-PE40, the control for sensitivity of the MLR to protein synthesis inhibition by PE40, was potently cytotoxic at 0.8 nM. In contrast, 1.7 nM sCD4-PE40 had negligible effect on the MLR of any of the 13 donors, and 17 nM sCD4-PE4 had negligible effect on the responses of 10 PBMC donors. 170 nM sCD4-PE40 had little effect on the MLR response of 4 of 13 donors, while a slight inhibition (30 to 60% of the control) was observed in the responses of the remaining 9 donors. Given the data presented in Fig. 1A, the mean inhibitory effect of 170 nM sCD4-PE40 on the MLR of 13 PBMC donors was well above a 50% inhibitory concentration (IC_{50}).

We next characterized the PBMC secondary response to TTOX as dependent on CD4 and MHC class II interaction by blocking studies with specific mAb in a manner similar to that reported above for the MLR (data not shown). PBMC from the 13 original donors were screened for proliferation in response to TTOX and we selected three responding donors for study with sCD4-PE40 (Fig. 1B). As in the MLR, 0.8 nM Tr-PE40 was potently cytotoxic for all three donors and the IC_{50} for Tr-PE40 in the TTOX assay was 0.08 nM.

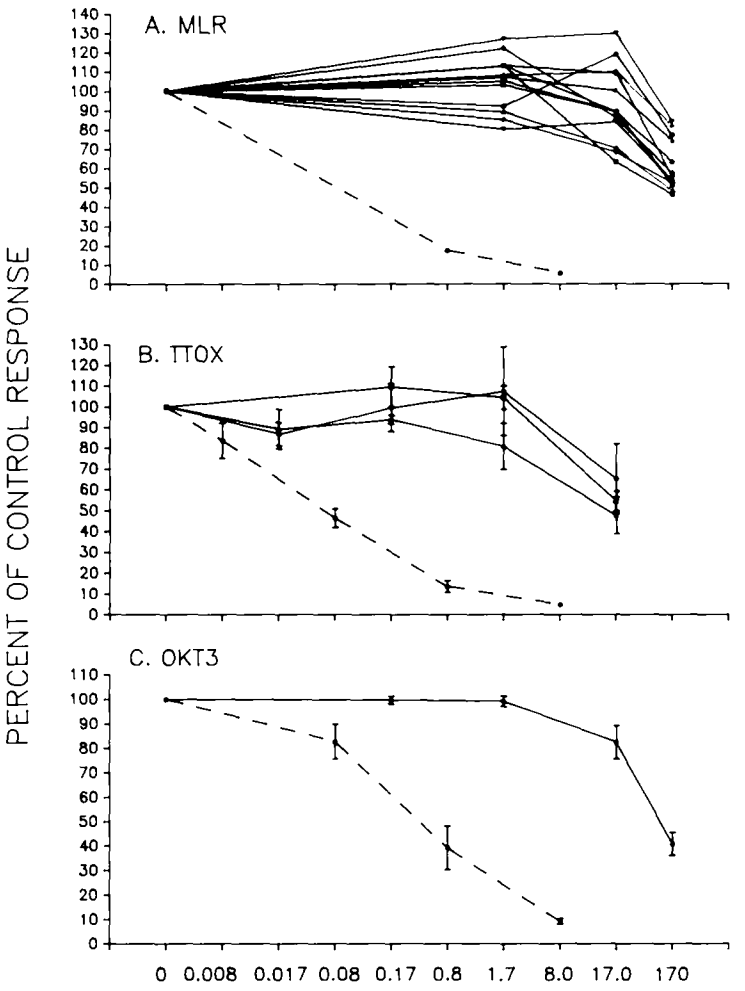

Fig. 1. Effects of sCD4-PE40 on PBMC proliferation. **A**: effects of sCD4-PE40 on alloantigen-stimulated PBMC proliferation. PBMC from 13 different donors were stimulated with allogeneic B-LCL in a one-way MLR and proliferation was measured by [^{3}H]thymidine uptake after 5 days of culture. sCD4-PE40 ( ) or Tr-PE40 (- - - -) were added at the time of initiation of culture. Values are expressed as the percentage of the control proliferative response to alloantigenic B-LCL in the absence of sCD4-PE40 or Tr-PE40 and proliferation inhibition was defined as any response which was less than 70% of the control. Data are from one representative experiment. **B**: effects of sCD4-PE40 on TTOX-stimulated PBMC proliferation. PBMC from 3 donors (a subset of the panel shown in Fig. 1A) were stimulated with TTOX and proliferation was measured by [^{3}H]thymidine uptake after 7 days of culture. sCD4-PE40 (: individual donors) or Tr-PE40 (- - - -) were added at the time of initiation of culture. Values are expressed as a percentage of the proliferative response to TTOX in the absence of sCD4-PE40 or Tr-PE40 and standard error of the mean (SEM) was derived from at least 2 independent experiments for each point. Inhibition was as described above for the MLR and data for proliferative responses to TTOX in the presence of Tr-PE40 were pooled from all 3 donors among experiments. **C**: effects of sCD4-PE40 on OKT3-stimulated PBMC proliferation. PBMC from 3 donors (as described in Fig. 1B) were stimulated with purified OKT3 and proliferation was measured by [^{3}H]thymidine uptake after 2 days in culture. sCD4-PE40 ( ) or Tr-PE40 (- - - -) were added at the time of initiation of culture. Data for individual donors were pooled and represent the mean and SEM from at least 2 independent experiments for each point for each donor. Values are expressed as a percentage of the proliferative response to OKT3 in the absence of sCD4-PE40 or Tr-PE40 with inhibition determined as for Fig. 1A and B.

In contrast, concentrations of sCD4-PE40 up to 1.7 nM had negligible effect on the TTOX response for any of the three donors. 17 nM sCD4-PE40 had negligible effect on the response of 1 of the 3 donors, while the responses of the remaining 2 donors were inhibited by 40 to 50%. 170 nM sCD4-PE40 was inhibitory for the TTOX response in all three donors tested (not shown). From the data presented in Fig. 1B, the IC_{50} for the chimeric sCD4-PE40 protein in the TTOX response was estimated to be 17 nM.

To observe the effect that sCD4-PE40 would have on a non-antigen dependent proliferative response, we investigated proliferation induced by crosslinking the CD3 component of the T cell receptor with anti-CD3 antibody (OKT3). Initially we observed that OKT3-induced proliferation was blocked by high concentrations of mAb to CD4 (Leu3a, 2.5 μg) and was inhibited by mAb to MHC class II (data not shown). Fig. 1C shows the effects of Tr-PE40 and sCD4-PE40 on the OKT3 proliferative responses of the same three PBMC donors tested in the TTOX assay. Tr-PE40 at 0.8 nM inhibited the OKT3 response by 60%, which was considerably less than that observed in the MLR and TTOX assays, and 8nM Tr-PE40 completely abrogated the OKT3 response. In contrast, concentrations of sCD4-PE40 up to 17 nM negligibly affected the PBMC response to OKT3; 170 nM sCD4-PE40 inhibited 50% of the response for the 3 donors thereby approximating the IC_{50}, for the OKT3 response (Fig. 1C).

Effect of sCD4-183 and sCD4-369 on PBMC proliferative responses

Increasing concentrations of sCD4-183 were evaluated for potential inhibitory effects on PBMC proliferation in response to MLR, TTOX and OKT3 (Fig. 2). Concentrations of sCD4-183 as high as 2.5 μM did not inhibit the response of the single donor in the MLR and similarly, negligibly affected the TTOX responses of 3 donors. In the antigen-independent system, 2.5 μM

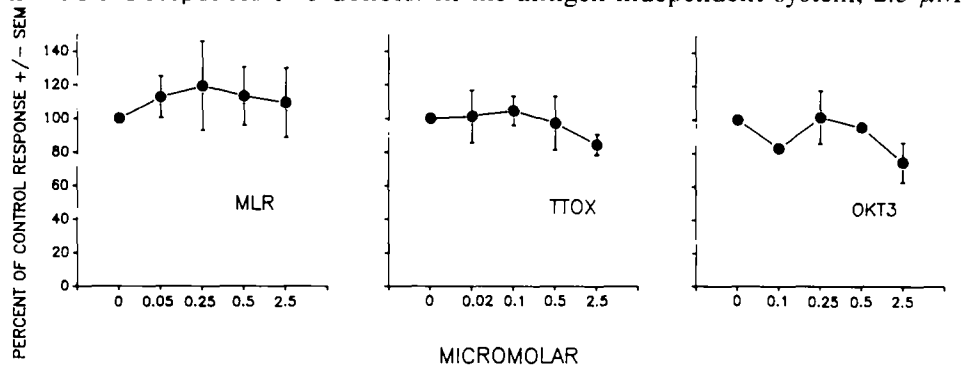

Fig. 2. Effects of sCD4-183 on PBMC proliferation. PBMC were stimulated with alloantigen (MLR, left panel), with TTOX (middle panel) or with OKT3 (right panel) and proliferation was measured by [^{3}H]thymidine incorporation. sCD4-183 was added at the time of initiation of culture. Values are expressed as a percentage of the proliferative response in the absence of sCD4-183 and represent the mean ± SEM of multiple experiments for a single donor (MLR) or multiple experiments for 3 or more donors (TTOX and OKT3). Inhibition was assessed as in Fig. 1.

sCD4-183 minimally affected OKT3-induced proliferation of PBMC from 3 donors (Fig. 2).

In a previously published report (Liu and Liu, 1989), a recombinant sCD4 containing 374 amino acids of the four extracellular domains expressed in mammalian cells did not have inhibitory activity for PBMC proliferation induced in MLR, TTOX or mitogen-driven assays at concentrations as high as 0.75 μM. For comparison purposes, we expressed in mammalian cells an analogous molecule containing the four extracellular domains, sCD4-369, and evaluated this molecule for potential inhibition of the MLR response in side-by-side experiments with sCD4-183. At the highest concentration tested, 1.2 μM, sCD4-369 had no significant effect on MLR-induced proliferation (data not shown). From these data, the IC_{50} for sCD4-183 was approximated as greater than 2.5 μM for the MLR, TTOX and OKT3 assays, and the IC_{50} for sCD4-369 was approximated as greater than 1.2 μM for the MLR.

Anti-HIV activity of sCD4s in PBMC

The anti-HIV activity of sCD4-PE40 was determined after the acute infection of primary cultures of human PBMC (Table 1). For these studies, triplicate cultures of PBMC were infected with the HIV_{D34} isolate and

TABLE 1

Anti-HIV activities of sCD4-PE40, sCD4-183 and sCD4-369

Treatment	Day 3		Day 4	
	ng HIV p24/ml[a]	%I[b,c]	ng HIV p24/ml	%I
sCD4-PE40 (nM)				
0	55.7	0	101.0	0
5.6	0	100	12.8	87
1.9	7	87	39.3	61
0.6	42.8	23	72.7	28
0.2	47.7	15	89.6	11
sCD4-183 (nM)				
0	192.7	0	236.6	0
1250	5.9	97	6.7	97
125	6.2	97	25.4	89
12.5	15.8	92	22.2	91
1.25	144.8	25	252.9	0
sCD4-369 (nM)				
0	192.7	0	236.6	0
600	7.2	96	18.5	92
60	9.6	95	13.1	94
6	125.8	35	237.9	0
0.6	200.7	0	239	0

[a]Levels of HIV p24 core protein released into supernatant fluids from HIV-infected PBMC were determined as described in Materials and Methods.

[b]% Inhibition was calculated as: $\frac{\text{ng HIV p24/ml from cultures with sCD4}}{\text{ng HIV p24/ml from untreated cultures}} \times 100$

[c]%I for sCD4-183 and sCD4-369 are reprinted from Garlick et al. (1990).

immediately exposed to sCD4-PE40. The extent of HIV replication in these cells was determined by quantitation of the levels of HIV p24 protein synthesized and released into the culture supernatant fluids at 3 and 4 days post infection. In Table 1, results are expressed both as the concentration of HIV p24 released (ng/ml) and the percentage of inhibition calculated as described for Table 1. In the control cultures which received no sCD4-PE40, the level of p24 antigen released into the supernatant increased two-fold from day 3 to day 4. Addition of 1.9 to 5.6 nM sCD4-PE40 to the culture reduced the levels of HIV p24 by 60 to 100% compared to levels found in untreated, infected culture supernatants. At 0.6 and 0.2 nM sCD4-PE40 the levels of p24 were reduced by 10 to 30% at both timepoints. From these data we estimated the IC_{50} of sCD4-PE40 in this assay to be approximately 1 nM, with a range between 0.6 and 1.9 nM. These concentrations of sCD4-PE40 (up to 5.6 nM) had no detectable effect on uninfected cultures, set up in parallel, when based on cell number or viability determined by trypan blue dye exclusion (data not shown).

In our recent work (Garlick et al., 1990) we investigated the anti-HIV activities of sCD4-183 and sCD4-369 under conditions identical to those described above for sCD4-PE40. These data are also shown in Table 1 with the addition of the raw data to the inhibitory values. In the control cultures which received no sCD4, the level of p24 antigen increased slightly from day 3 to day 4. Addition of 12.5 nM sCD4-183 reduced p24 levels in HIV-infected PBMC culture supernatants by at least 90% at both days 3 and 4 compared to levels in untreated, infected controls. From these and additional data in Table 1, we estimated the IC_{50} for sCD4-183 to be approximately 7 nM, with a range between 1.25 and 12.5 nM. Addition of at least 60 nM sCD4-369 was required for 90% reduction of p24 antigen levels at days 3 and 4 post infection (Table 1). Addition of 6 nM sCD4-369 reduced p24 antigen levels by 35% at day 3 post infection but this antiviral effect was not apparent at 4 days post infection. From these and additional data in Table 1, we estimated the IC_{50} of sCD4-369 to be approximately 30 nM, with a range between 6 and 60 nM.

Estimation of the selectivity indices for sCD4-PE40, sCD4-183 and sCD4-369

Using data from the proliferation and antiviral assays described above we estimated the selectivity index for each sCD4-based proteins. The tested concentration of drug which most closely approximated an IC_{50} in the individual proliferation assay was divided by the IC_{50} estimated from the antiviral assay to yield the estimated indices shown in Table 2.

Discussion

These studies evaluated in vitro characteristics of 3 different sCD4-based proteins, sCD4-PE40, CD4-183, and sCD4-369 on human PBMC, and compared their distinctive antiviral potencies with their specific inhibitory

TABLE 2

Estimation of the selectivity indices for sCD4-PE40, sCD4-183 and sCD4-369

A. Summary of IC_{50} determinations from proliferation and antiviral assays[a]

Assay	sCD4-PE40	sCD4-183	sCD4-369
MLR	0.170 μM	$\geq$2.5 μM	$\geq$1.2 μM
TTOX	0.017 μM	$\geq$2.5 μM	ND[b]
OKT3	0.170 μM	$\geq$2.5 μM	ND
HIV infectivity	1 nM (0.6–0.9 nM)[c]	7 nM (1.25–12.5 nM)	30 nM (6–60 nM)

B. Selectivity indices[d]

Assay	sCD4-PE40	sCD4-183	sCD4-369
MLR	170	$\geq$350	$\geq$40
TTOX	17	$\geq$350	
OKT3	170	$\geq$350	

[a]IC_{50} values were determined with respect to toxicity in the proliferation assays and efficacy in the anti-HIV assay as described in Results.
[b]Not done.
[c]Values in parentheses denote range of IC_{50} obtained from antiviral assay. The approximate midpoint of each range was estimated for each sCD4.
[d]Selectivity indices were calculated as: proliferative toxicity/antiviral efficacy

properties for antigen-stimulated lymphocyte activation. Our data revealed that sCD4-PE40, sCD4-183 and sCD4-369 each had potent antiviral activity for PBMC infected with HIV_{D34}, and that the antiviral activity was manifest at concentrations which were well below those which endangered human T-cell activation. We observed antiproliferative activity of sCD4-PE40 at concentrations which were 10 to 100-fold greater than those which were antiviral in our assays. The highest concentration of sCD4-183, 2.5 μM, which minimally affected our proliferation assays was 200-fold greater than that required for antiviral activity; similarly, the highest concentration of sCD4-369, 1.2 μM, which minimally affected the MLR, was 20-fold greater than that required for antiviral activity. While we recognize that laboratory strains of HIV may be uniquely sensitive to sCD4-mediated antiviral effects (Daar et al., 1990), the design of our assay systems allowed us to evaluate the 3 different sCD4s in primary cultures of human PBMC and to directly compare the *E. coli*-produced sCD4-183 with the mammalian cell-produced sCD4-369. Such relationships among sCD4s have not been previously evaluated. Furthermore, the antiviral potencies of other sCD4s have been frequently evaluated with the HTLV III_B isolate, thus allowing for some degree of cross-comparison of data.

As measured by p24 antigen released into the supernatants of infected cells, antiviral activity of sCD4-PE40 for acutely-infected PBMC was observed at concentrations as low as 0.2 nM, having an estimated IC_{50} of 1 nM. These results confirm and extend those of Chaudhary et al. (1988) who demonstrated that protein synthesis in CV-1 cells expressing gp120 and in the human 8E5 cell line chronically infected with HIV-1_{LAV} was inhibited by 50% at 0.45 nM and

1.6 nM of sCD4-PE40, respectively. Taken together, these data suggest that the antiviral potency of sCD4-PE40 may be equivalent among different in vitro assay systems having different endpoints. Using the PMBC proliferative response to alloantigen, to TTOX and to crosslinking with OKT3 as an in vitro correlate for the potential inhibitory effects of sCD4-PE40 on primary and secondary immune responsiveness to antigen, selectivity indices of 170, 17 and 170, respectively, were estimated for sCD4-PE40 (Table 2). Our previous studies demonstrated that addition of 17 nM sCD4-PE40 had no effect on the alloantigen (MLR) response of PBMC from a single donor and no effect on the proliferative response of a human T-cell clone specific for influenza hemagglutinin (Berger et al., 1990). Herein, we confirmed and extended these results with PBMC from multiple donors at increasing concentrations of sCD4-PE40. Our data revealed that the secondary immune response to TTOX, and presumably other nominal antigens, by non-cloned PBMC may be most sensitive to potential inhibitory effects of sCD4-PE40. The increased sensitivity of the TTOX response, compared to the response to alloantigen or crosslinking with OKT3, may be a consequence of evoking responses in different subsets of human T cells. For example, 'naive' cells respond to alloantigen and 'memory' cells to TTOX (Sanders, 1989); OKT3-responding cells would be expected to encompass both subtypes of T cells. Alternatively, the increased sensitivity of the TTOX response to sCD4-PE40 inhibition may reflect the relative number of T cells responding to antigen in that the response to alloantigen occurs in an unusually large number of T-cell precursors (Lechler et al., 1990) whereas the response to TTOX occurs only in a relatively small, antigen-primed subset of T cells; and OKT3 would transduce activation signals in essentially all T-cell-receptor-bearing PBMC.

sCD4-183, produced from *E. coli*, and mammalian-cell-derived recombinant sCD4-369, were also evaluated for antiviral and anti-proliferative activities. The latter molecule, sCD4-369 modeled on all four extracellular domains of the natural ligand, was prepared for comparative purposes as an analog of several previously described recombinant sCD4s produced from mammalian cells (Deen et al., 1988; Smith et al., 1987; Fisher et al., 1988) or from baculovirus expression vectors (Hussey et al., 1988) all of which have been reported to have potent antiviral activity in vitro, similar to that determined in our studies for sCD4-PE40 and sCD4-183. One of these sCD4 (Fisher et al., 1988) has also been extensively studied for potential inhibitory effects on in vitro human PBMC proliferation and found to have negligible effect at 30 μg/ml (approximately 0.75 μM) in systems analogous to ours (Liu and Liu, 1988). Not surprisingly, our studies revealed that sCD4-183 and sCD4-369 had negligible, if any, effect on human PBMC proliferation at concentrations as high as 2.5 or 1.2 μM, respectively. However, measurement of the antiviral potencies of these sCD4s against HIV-infected PBMC in parallel experiments revealed significant differences in their respective IC_{50}s, estimated to be 7 nM for sCD4-183 compared to 30 nM for sCD4-369 (Table 2 and Garlick et al., 1990). These data validate a direct comparison between the two sCD4s and

suggest that the relative selectivity index of sCD4-183 may be greater than that of its mammalian cell-derived, full-length extracellular counterpart, sCD4-369. The reason for this apparent discrepancy in antiviral potency is not obvious, particularly since glycosylation of CD4 does not appear to play a significant role in binding gp120 in vitro (Fenouillet et al., 1989). In this regard, it will be of interest to directly compare antiviral potencies of sCD4-183 from *E. coli* with truncated forms of sCD4 produced from mammalian cells against a variety of isolates of HIV. Since the levels of HIV infection in the sCD4-183 and sCD4-369 antiviral assays were 2 to 3.5-fold higher than in the sCD4-PE40 antiviral assay, the selectivity indices estimated for sCD4-PE40 cannot be directly compared with those of sCD4-183 and sCD4-369.

The data reported herein may be helpful in making predictions about drug levels in serum which may be required for antiviral activity without overtly jeopardizing cells which bear the natural ligand for CD4. However, evaluation of the sCD4s against multiple primary viral isolates of HIV will be required to accurately estimate the in vivo therapeutic potential of each of these compounds. While the absolute selectivity index estimations may vary depending on the specific viral isolate, we do not expect the relative indices of these sCD4s to change.

Acknowledgements

The authors wish to thank Dr. Martin Sanders for providing leukapheresis packs as a source of PBMC and Dr. Robert Garlick for production of sCD4s.

References

Berger, E.A., Fuerst, T.R. and Moss, B. (1988) A soluble recombinant polypeptide comprising the amino-terminal half of the extracellular CD4 molecule contains an active binding site for human immunodeficiency virus. Proc. Natl. Acad Sci. USA 85, 2357–2361.

Berger, E.A., Chaudhary, V.K., Clouse, K.A., Jaraquemada, D., Nicholas, J.A., Rubino, K.L., FitzGerald, D.J., Pastan, I. and Moss, B. (1990) Recombinant CD4-*Pseudomonas* exotoxin hybrid protein displays HIV-specific cytotoxicity without affecting MHC class II dependent functions. AIDS Res. Hum. Retroviruses 6, 795–804.

Chaudhary, V.K., Mizukami, T., Fuerst, T.R., FitzGerald, D.J., Moss, B., Pastan, I. and Berger, E.A. (1988) Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* exotoxin hybrid protein. Nature 335, 369–372.

Clapham, P.R., Weber, J.N., Whitby, D., McIntosh, K., Dalgleish, A.G., Maddon, P.J., Deen, K.C., Sweet, R.W. and Weiss, R.A. (1989) Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells. Nature 337, 368–370.

Deen, K.C., McDougal, J.S., Inacker, R., Folena-Wasserman, G., Arthos, J., Rosenberg, J., Maddon, P.J., Axel, R. and Sweet, R.W. (1988) A soluble form of CD4(T4) protein inhibits AIDS virus infection. Nature 331, 82–84.

Farrar, W.L., Harel-Bellan, A. and Ferris, D.K. (1988) Characterization of CD4 glycoprotein determinant-HIV envelope protein interactions: perspectives for analog and vaccine development. Crit. Rev. Immunol. 8, 315–339.

Fenouillet, E., Clerget-Raslain, B., Gluckman, J.C., Guetard, D., Montagnier, L. and Bahraoui, E. (1989) Role of *N*-linked glycans in the interaction between the envelope glycoprotein of human immunodeficiency virus and its CD4 cellular receptor: structural enzymatic analysis. J. Exp. Med. 169, 807 822.

Fisher, R.A., Bertonis, J.M., Meier, W., Johnson, V.A., Costopoulos, D.S., Liu, T., Tizard, R., Walker, B.Z., Hirsch, M.S., Schooley, R.T. and Flavell, R.A. (1988) HIV infection is blocked in vitro by recombinant soluble CD4. Nature 331, 76 78.

Garlick, R.L., Kirschner, R.J., Eckenrode, F.M., Tarpley, W.G. and Tomich, C.-S.C. (1990) *E. coli* expression, purification and biological activity of a truncated soluble CD4. AIDS Res. Hum. Retroviruses 6, 465–480.

Hussey, R.E., Richardson, N.E., Kowalski, M., Brown, N.R., Chang, H-C., Siliciano, R.F., Dorfman, T., Walker, B., Socroski, J. and Rehinherz, E.L. (1988) A soluble CD4 protein selectively inhibits HIV replication and syncytia formation. Nature 331, 78 81.

Issekutz, T., Chu, F. and Geha, R.S. (1982) Antigen presentation by human B cells: T cell proliferation induced by Epstein-Barr virus B lymphoblastoid cells. J. Immunol. 129, 1446 1450.

Janeway, C.A. (1989) The role of CD4 in T-cell activation: accessory molecule or co-receptor? Immunol. Today 10, 234–238.

Kahn, J.O., Allan, J.D., Hodges, T.L., Kaplan, L.D., Arri, C.J., Fitch, H.F., Izu, A.E., Mordenti, J., Sherwin, S.A., Groopman, J.E. and Volberding, P.A. (1990) The safety and pharmacokinetics of recombinant soluble CD4 (rCD4) in subjects with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex: a phase I study. Ann. Intern. Med. 112, 254 261.

Lechler, R.L., Lombardi, G., Batchelor, R., Reinsmoen, N. and Bach, F.H. (1990) The molecular basis of alloreactivity. Immunol. Today 11, 83 88.

Liu, M.A. and Liu, T. (1988) Effect of recombinant soluble CD4 on human peripheral blood lymphocyte responses in vitro. J. Clin. Invest. 82, 2176 2180.

McQuade, T.J., Pitts, T.W. and Tarpley, W.G. (1989) A rapid solution immunoassay to quantify binding of the human immunodeficiency virus envelope glycoprotein to soluble CD4. Biochem. Biophys. Res. Commun. 163, 172-176.

Rubsamen-Waigmann, H., Becker, W.-B., Helm, E.B., Brodt, R., Fischer, H., Henco, K. and Brede, H.D. (1986) Isolation of variants of lymphocytopathic retroviruses from the peripheral blood and cerebrospinal fluid of patients with ARC or AIDS. J. Med. Virol. 19, 335 344.

Schooley, R.T., Merrigan, T.C., Gaut, P., Hirsch, M.S., Holodnly, M., Flynn, T., Liu, S., Byington, R.E., Henochowicz, S., Gubish, E., Spriggs, D., Kufe, D., Schindler, J., Dawson, A., Thomas, D., Hanson, D.G., Letwin, B., Liu, T., Gulinello, J., Kennedy, S., Fisher, R. and Ho, D. (1990) Recombinant soluble CD4 therapy in patients with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex: a phase I-II escalating dosage trial. Ann. Intern. Med. 112, 247 253.

Smith, D.H., Byrn, R.A., Marsters, S.A., Gregory, T., Groopman, J.E. and Capon, D.J. (1987) Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. Science 238, 1704 1707.

Traunecker, A., Luke, W. and Karjalainen, K. (1988) Soluble CD4 molecules neutralize human immunodeficiency virus type 1. Nature 331, 84 86.

von-Briesen, H., Becker, W.-B., Henco, K., Helm, E.B., Gelderblom, H., Brede, H.D. and Rubsamen-Waigmann, H. (1987) Isolation frequency and growth properties of HIV variants: multiple simultaneous variants in a patient demonstrated by molecular cloning. J. Med. Virol. 23, 51–66.