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### Research Article

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## Effect of Recombinant Soluble CD4 on Human Peripheral Blood Lymphocyte Responses In Vitro

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### Abstract

We have previously demonstrated that recombinant soluble CD4 protein (rsT4) blocks both HIV-1 infection of CD4 bearing lymphocytes and syncytium formation in vitro. (Recombinant soluble CD4 is designated by rsT4.) Hence, we suggested the use of rsT4 in therapy for AIDS or the prevention of HIV-1 infection in individuals with a known risk of exposure. However, concerns arose that rsT4 might be immunosuppressive because of its implicated role in the enhancement of certain lymphocyte activation events through its engagement of MHC class II molecules on target cells. We therefore assessed the effect of recombinant soluble CD4 upon a number of functional and activation parameters of lymphocytes, including cellular proliferation, IL-2 secretion, and cytolytic capability, after antigenic or mitogenic stimulation. We report here that rsT4, at 60-fold over the concentration needed to block acute HIV-1 infection in vitro, does not significantly inhibit the activation of human peripheral blood lymphocytes by either PHA, tetanus toxoid or allogeneic cells. These results indicate that rsT4 will potentially exert minimal immunosuppressive effects in vivo, thus supporting the feasibility of clinical trials of rsT4 in the treatment or prevention of AIDS. In addition, the implications of these results for the interactions between CD4 and MHC class II molecules during lymphocyte activation are discussed.

### Introduction

Human immunodeficiency virus type 1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (AIDS), infects cells by binding to a cell surface molecule, the CD4 antigen, present on certain cells of the immune system (1-5). In particular, the CD4 antigen has served as a phenotypic marker for those T lymphocytes that recognize antigen on cells bearing MHC class II molecules (6-10). Functionally, most of the CD4<sup>+</sup> lymphocytes in human peripheral blood are helper/inducer cells, although CD4<sup>+</sup> cytotoxic cells have been detected (11). The cell surface CD4 molecule has been thought to en-

hance the interactions between the T lymphocyte and the antigen presenting cell by binding to monomorphic determinants on the MHC class II molecule (12-17).

Recombinant soluble CD4 protein (lacking its transmembrane and cytoplasmic domains) has recently been expressed and shown to be capable of inhibiting virus infection and HIV-1-induced syncytium formation in vitro (18-22). These findings have led to proposals for the use of recombinant soluble CD4 (rsT4)<sup>1</sup> as an antiviral therapy for HIV-1 infection. Concerns exist however, that rsT4 could act as an immunosuppressant by competing with cell surface CD4 for binding to its putative ligand, MHC class II molecules, and as a result compromise activation of CD4<sup>+</sup> lymphocytes. Partial inhibition of lymphokine production and cytotoxic activity by anti-CD4 and anti-class II antibodies in several systems supports such concerns (12, 13, 17, 23).

To determine if rsT4 has immunosuppressive properties, we examined the peripheral blood lymphocyte responses to three types of antigenic and mitogenic stimuli in vitro in the presence and absence of rsT4 or control antibodies. We utilized freshly isolated human PBL as the responder cells in order to simulate the physiologic milieu and responses. The three types of stimuli were allogeneic cells, soluble antigen, and mitogenic lectin. The responses of the peripheral blood lymphocytes that were examined included proliferation, cell surface phenotype, IL-2 production, and cytolytic capability.

### Methods

#### *Peripheral blood lymphocytes and cell lines*

Fresh PBL were obtained from six healthy donors by isolating heparinized venous blood on Ficoll-Paque. All of the donors had normal CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratios, and none were in any high risk category for HIV-1 infection. The lymphocytes were washed twice in RPMI and resuspended at  $2 \times 10^6$  cells/ml and  $1 \times 10^6$  cells/ml in K medium (RPMI 1640 supplemented with 10% FCS, 50  $\mu$ M 2-mercaptoethanol, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, and 10 mM Hepes). The lymphoblastoid B cell line JY, was maintained in K media. Before use, the JY cells were washed and resuspended at  $2 \times 10^6$  cell/ml in K medium then irradiated with 6,000 rad.

#### *rsT4 and antibodies*

rsT4 was purified from conditioned medium of CHO cells expressing BG 381. The purified protein was generously provided by W. Meier and J. Maraganore, Biogen. Antibodies directed against CD4 were included as controls: OKT4a, an anti-CD4 antibody known to inhibit

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1. Abbreviations used in this paper: rsT4, designation for recombinant soluble CD4.

Table 1. Effect of Anti-CD4 Antibodies, Control Ab, and Recombinant Soluble CD4 upon Mixed Lymphocyte Proliferation

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Control	41,056±890	18,035±339	27,460±1,303	26,914±2,494	22,300±2,363	30,666±1,164
81AB7	34,019±787	15,547±775	25,481±1,802	21,514±452	25,348±1,617	29,739±1,845
OKT4a	16,205±919	8,668±515	12,752±403	8,476±301	14,215±648	16,623±973
OKT4	19,997±1,279	13,360±801	14,663±450	11,221±1,976	15,295±392	20,332±883
rsT4	31,547±1,463	14,662±253	23,258±1,330	20,865±879	23,845±2,853	29,512±1,363

The proliferation of PBL derived from six normal donors versus JY B lymphoblastoid cells was assessed by [<sup>3</sup>H]thymidine incorporation on day 3 of the MLR. The effect of control antibody 81AB7 (5 µg/ml), 2 anti-CD4 antibodies (OKT4a, OKT4, both 5 µg/ml), and recombinant soluble CD4 (rsT4, 30 µg/ml) added to the MLR at the time of initiation is shown. The numbers are average counts per minute of triplicate assays±1 SD. Recombinant soluble CD4 (rsT4) has a minimal inhibitory effect at 30 µg/ml, whereas OKT4a and OKT4 have marked inhibitory effects at 5 µg/ml.

a variety of T cell responses (11, 23–25), was purchased from Ortho-mune; OKT4 was purified conventionally from the conditioned medium of OKT4 hybridoma cells purchased from American Type Culture Collection. 81AB7, a gift from D. Hess (Biogen Research Corp.) was selected to control for any effect due to the IgG2a Fc region of OKT4a. It is specific for bee venom phospholipase A2 with no known cross-reactivity with human phospholipase.

#### Stimulation of peripheral blood lymphocytes

**Mixed lymphocyte responses (MLR).** In 96 well Costar microtiter plates, wells containing  $1 \times 10^5$  PBL and  $1 \times 10^5$  JY in 200 µl of K medium were set up in triplicate. Samples for baseline proliferation contained no JY; control MLRs contained PBL and JY. Other samples contained, in addition to the PBL and JY, OKT4a (IgG2a, 5 µg/ml), OKT4 (IgG2b, 5 µg/ml), rsT4 (30 µg/ml), or 81AB7 (IgG2a, 5 µg/ml).

**Specific soluble antigenic stimulation.** Samples in triplicate contained  $2 \times 10^5$  PBL, 50 µg/ml tetanus toxoid with or without 81AB7, OKT4a, OKT4, or rsT4 (at the concentrations given above) in 200 µl of K medium. Donors 1–3 had been boosted with tetanus toxoid 7–8 d before blood donation as none of them had received the toxoid within 10 y. Data derived from donor 4 was excluded since he had never received a complete primary immunization series, resulting in extremely low proliferation (1,500 cpm).

**Mitogenic activation.** PBL were cultured in triplicate samples in 200 µl of K medium with 5 µg/ml of PHA (Difco Laboratories, Detroit, MI) in the presence and absence of 81AB7, OKT4a, OKT4, or rsT4 at the concentrations specified above.

**Dose-response effect.** Mixed lymphocyte responses were generated between  $1 \times 10^5$  PBL and  $1 \times 10^5$  JY cells per well in the presence and absence of 81AB7, OKT4a, OKT4, and rsT4 at the concentrations noted above as well as the following dilutions: 1:10, 1:10<sup>2</sup>, 1:10<sup>4</sup>, 1:10<sup>6</sup>, and 1:10<sup>8</sup> in triplicate in a final volume of 200 µl of K medium.

#### Quantitation of IL-2 production

50 µl of supernatant was harvested from cultures of PBL from donors 1, 2, and 6 that had been exposed to tetanus toxoid in the presence and absence of rsT4 and the control antibodies. IL-2 production was measured by standard CTLL proliferation assays (15).

#### PBL proliferation assays

At the time specified below, 100 µl of K medium containing 1 µCi of [<sup>3</sup>H]thymidine was added to each well. Cells were harvested 17 h later with a Skatron harvester and filter disks placed in 1 ml of beta-fluor and counted in a Beckman gamma counter. The counts per minute for each sample is calculated as the mean of the triplicate values. The data for the day 3 MLR proliferation ± 1 SD are shown in Table I. In all the figures, the mean triplicate values for each donor were calculated as a percentage of the control using the equation (E-S)/(C-S), where E is the experimental [<sup>3</sup>H]thymidine incorporation (cpm) in the presence of

the antibody or protein, S the [<sup>3</sup>H]thymidine incorporation (cpm) of PBL alone, and "C" the [<sup>3</sup>H]thymidine incorporation of the control PBL plus stimulus (no antibody or rsT4 present). These percent control values for the six donors were then averaged to yield the mean values. Proliferation assays were done at 72, 96, and 120 h for the MLRs, at 96 h for the MLR with dilutions of rsT4 and antibodies, at 96 h for the tetanus toxoid stimulation, and at 48 and 72 h for the PHA activation, since these are the optimal response times for the various stimuli.

#### Phenotype analysis

Phenotype of the responder cells were analyzed by flow cytometry on day zero (before initiation of the MLR), and again at day 4 or day 5 using two-color analysis with fluorescein-labeled anti-CD4 (FITC-

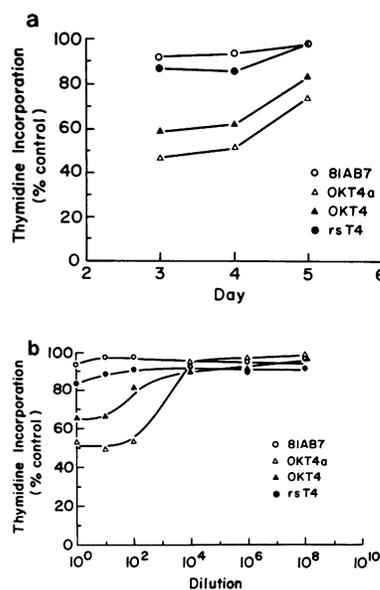
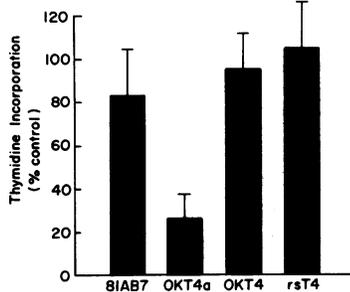


Figure 1. (a) MLR between peripheral blood lymphocytes from six normal donors and JY cells were established in the absence and presence of control antibody (81AB7), anti CD4 antibodies (OKT4a, OKT4) and recombinant soluble CD4 (rsT4) (see Table I legend). The average triplicate value was expressed as a percentage of the control for each donor and these percent control values for the six donors were then averaged to yield the mean values which are plotted here for days

3–5. rsT4 at concentration of 30 µg/ml has a minimal inhibitory effect upon proliferation for days 3 and 4, whereas the effect of OKT4a and OKT4 at 5 µg/ml is more marked for all 3 d. (b) The MLR were established as before. The same initial concentrations of antibodies 81AB7, OKT4a, OKT4 (5 µg/ml), and rsT4 (30 µg/ml) were used as well as 10-fold dilutions of each. On day 4 the proliferation was assessed by thymidine incorporation and the values expressed as percentages of the control proliferation as described in a. The minimal inhibitory effect of rsT4 is seen only at the highest 30 µg/ml concentration, whereas the greater inhibitory effects of OKT4a and OKT4 persist to 50 ng/ml.



**Figure 2.** The effect of 81AB7, OKT4, OKT4a, and rsT4 upon tetanus toxoid induced proliferation of peripheral blood lymphocytes from five normal donors. Peripheral blood lymphocytes from five normal donors were stimulated with 50  $\mu\text{g}/\text{ml}$  tetanus toxoid in the presence and absence of antibodies (5

$\mu\text{g}/\text{ml}$ ) or rsT4 (30  $\mu\text{g}/\text{ml}$ ). Proliferation was assayed on day 4 by [ $^3\text{H}$ ]thymidine incorporation and the results of the cultures containing antibody or rsT4 were expressed as a percentage of the control for each individual, which were then averaged to yield the mean percent control for each experimental condition. rsT4 had no net effect whereas OKT4a had a marked inhibitory effect upon proliferation.

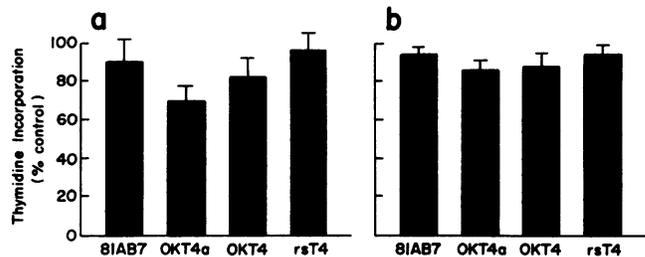
Leu3a) and phycoerythrin-labeled anti-CD8 (PE-Leu2a). On day 0 and day 4 for donors 1–3, and day 5 for donors 4–6,  $1 \times 10^5$  cells from each MLR were placed in duplicate test tubes, centrifuged, then resuspended in 50  $\mu\text{l}$  of medium containing PBS (0.14 M NaCl, 1 mM KCl, and 3.7 mM sodium-potassium phosphate), 5% FCS and 0.02% sodium azide. 30  $\mu\text{l}$  of control antibody or FITC-Leu3a/PE-Leu2a (Simulstest Immune Monitoring Kit; Becton Dickinson, Mountain View, CA) was added. After 30 min incubation on ice in the dark, the cells were fixed with 3% formaldehyde before analysis on a Becton Dickinson FACS Star flow cytometer.

#### Cytolytic assays

Standard 4-h  $^{51}\text{Cr}$  killing assays were performed using MLR responder cells from two donors at an E:T ratio of 10:1 in the presence and absence of rsT4 or the antibodies at their usual concentrations.

## Results

Table I and Fig. 1 *a* show the extent of PBL proliferation in an MLR in the presence of 30  $\mu\text{g}/\text{ml}$  of rsT4. This rsT4 concentration far exceeds the 0.5  $\mu\text{g}/\text{ml}$  level needed to inhibit HIV-1 replication in acutely infected H9 cells as measured by p24 antigen production (Johnson, V. A., personal communication). In contrast, 5  $\mu\text{g}/\text{ml}$  of OKT4a or OKT4 caused marked inhibition of  $\sim 50\%$  on day 3. The mean values of triplicate samples (with their standard deviations) for day 3 of the MLR are given in Table I to illustrate the magnitude of the response for the individual donors; the relative effect of each antibody or rsT4 among the individuals was reasonably consistent. These values were then expressed as a percentage of the control MLR for each individual, averaged to yield the mean from the six donors and are shown in Fig. 1 *a* for days 3, 4, and 5. The dose-response curves in Fig. 1 *b* show that the inhibitory effect of the two anti-CD4 antibodies diminished with decreasing concentration. A slight inhibitory effect of rsT4 was discernible at 30  $\mu\text{g}/\text{ml}$ . Extension of the dose range of rsT4 to 200  $\mu\text{g}/\text{ml}$  using PBLs from two other healthy donors did not result in inhibition of MLR proliferation. The level of rsT4 as detected by a sandwich ELISA using both OKT4 and OKT4a monoclonal antibodies, and the functional activity as measured by binding to recombinant HIV-1 envelope glycoprotein, gp120, remained the same before and after 3 and 4 d in tissue culture in a MLR indicating that extensive proteolysis or sequestration did not occur.



**Figure 3.** The effect of 81AB7, OKT4a, OKT4, and rsT4 upon mitogen-induced proliferation of peripheral blood lymphocytes from six normal donors. Peripheral blood lymphocytes were stimulated with 5  $\mu\text{g}/\text{ml}$  of phytohemagglutinin in the presence and absence of antibodies (5  $\mu\text{g}/\text{ml}$ ) or rsT4 (30  $\mu\text{g}/\text{ml}$ ). Proliferation was assayed at 48 h (*a*) and 72 h (*b*) and the results were calculated as in Fig. 2. rsT4 caused no significant inhibition, and OKT4a and OKT4 were moderately antiproliferative early on.

In addition, rsT4 did not affect the phenotype distribution or the cytolytic activity of cells in the MLR cultures. Analysis of the MLR responder cells by fluorescent flow cytometry demonstrated that the presence of rsT4, OKT4, or 81AB7 during generation of the MLR did not consistently alter the CD4/CD8 ratios of the cells from that of the control MLR (data not shown). In the presence of OKT4a,  $< 0.02\%$  of the cells were CD4 $^+$ . (This appears to be partially caused by blocking of FITC-Leu3a staining by persistent OKT4a in the culture medium.) Standard  $^{51}\text{Cr}$  release cytolytic assays of responder cells derived from the control MLR showed that the presence of rsT4 or the antibodies did not alter the lytic ability of the cells which averaged 34% specific lysis of JY at an effector to target cell ratio of 10:1.

The proliferative response of PBL to tetanus toxoid (50  $\mu\text{g}/\text{ml}$ ) was on average unaffected by rsT4 (Fig. 2), even though this stimulation is MHC class II restricted and selectively activates T cells which are CD4 $^+$  (26–28). The range for the individual donors varied from 82 to 130% of the respective controls, the mean being 105%. OKT4a was highly inhibitory but OKT4 had no significant effect. In addition, when IL-2 production by cells from donors 1, 2, and 6 was examined, the cultures stimulated in the presence of rsT4 varied from 81 to 140% of control on the day of optimal production, while OKT4a containing cultures were all completely inhibited. The variation as well as the extent of proliferation of the control cultures were not related to whether the individual had been boosted with tetanus toxoid the week before blood donation.

rsT4 had no effect on the generalized mitogenic effect of PHA for mature human T cells. As previously demonstrated (29), both OKT4a and OKT4 were less effective in inhibiting this PBL response than the MLR-induced proliferation. The maximal antibody effect was seen on day 2, with a smaller effect on day 3 after the one-time addition of PHA (5  $\mu\text{g}/\text{ml}$ ) on day zero (Fig. 3).

## Discussion

Our experiments show that rsT4 does not significantly inhibit human PBL responses to allogeneic cell, tetanus toxoid stimulation, or PHA in vitro. The use of PBL rather than T cell clones (19) allowed us to assess responses to primary as well as secondary stimuli. The parameters we measured in all three responses were unaffected by rsT4 within the limit of variation between the six individual donors except for the slight mean

effect at 30  $\mu\text{g/ml}$  of rsT4, on the MLR proliferation. However, no inhibitory effect was observed when the experiments were extended to 200  $\mu\text{g/ml}$  of rsT4. Under these experimental conditions, rsT4 remained intact physically with respect to the epitopes recognized by both OKT4 and OKT4a monoclonal antibodies, and it retained an active binding site for recombinant HIV-1 envelope glycoprotein, gp120. The lack of inhibitory effect by rsT4 in these systems could simply be attributed to the possibility that rsT4 is incapable of competing with membrane-bound CD4 for its physiological ligand even though it can bind to HIV gp160/120 and can be immunoprecipitated by both OKT4 and OKT4a antibodies (data not shown). Alternatively, it is possible that the CD4/MHC class II interaction only plays a prominent role under special conditions. Indeed, the enhancement of lymphocyte activation through CD4/MHC class II interaction was best demonstrated in a model system using cells with a lower level of T cell receptors on the surface (17). A third possibility is that CD4 facilitates lymphocyte response by acting as a ligand for MHC class II molecules on antigen-presenting cells. If soluble CD4, like membrane-bound CD4, binds to class II molecules, rsT4 would participate in, rather than inhibit, the immune response.

While the physiological role of CD4 remains to be defined, the observation that rsT4 does not significantly inhibit the immune responses of peripheral blood lymphocytes induced by several different stimuli provides encouragement for its use in the therapy of AIDS, especially if the syndromes of primary HIV infection can be recognized, as recently described (30). These findings also raise interesting questions about the interactions between CD4<sup>+</sup> T lymphocytes and MHC class II bearing antigen-presenting cells.

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