Selective Elimination of HIV-1–Infected Cells with an Interleukin-2 Receptor–Specific Cytotoxin

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Infection by human immunodeficiency virus type 1 (HIV-1) is associated with cellular activation and expression of the interleukin-2 (IL-2) receptor. A genetically engineered fusion toxin, DAB_{486} IL-2, that contains the enzymatic site and translocation domain of diphtheria toxin and the receptor binding domain of IL-2 specifically kills cells that express high-affinity IL-2 receptors. This toxin selectively eliminated the HIV-1–infected cells from mixed cultures of infected and uninfected cells and inhibited production of viral proteins and infectious virus. Thus, cellular activation antigens present a target for early antiviral intervention.

HIV-1 IS A LYMPHOTROPHIC RETROVIRUS that initially may cause T cell activation (*1, 2*) but ultimately leads to a fatal collapse of the immune system. Interaction of HIV-1 with its cell surface receptor, CD4, results in secretion of cytokines and de novo expression of activation antigens on T cells and monocytes (*2–4*). Binding of gp120 (from HIV-1) alone leads to IL-2 receptor (IL-2R) expression in monocytes (*4*). The cross-linking of CD4 leads to IL-2R expression in T cells, but the mechanism by which HIV-1 infection is associated with IL-2R expression is not defined (*5, 6*). It is clear, however, that the virus replicates in activated T cells and not in quiescent T cells (*7–9*).

Diphtheria toxin (DT), an exotoxin of *Corynebacterium diphtheriae*, kills eukaryotic cells by catalyzing the nicotinamide adenine dinucleotide–dependent adenosine diphosphate (ADP)–ribosylation of eukaryotic elongation factor 2 (EF-2). The ADP-ribosylation of EF-2 inhibits protein synthesis, which causes cell death. The DT molecule comprises an enzymatically active fragment A and a B fragment that has the eukaryotic receptor binding domain and cell membrane translocation sequences that deliver fragment A to the cell cytosol. By genetically replacing the native DT receptor binding domain with IL-2 sequences, researchers made a fusion toxin (DAB_{486} IL-2) that was selective for cells that express the high-affinity IL-2R (*10, 11*). DAB_{486} IL-2 specifically targets and kills IL-2R–expressing tumor cells and activated lymphocytes; the fusion toxin is useful in the control of graft rejection and certain autoimmune disorders because it is selectively cytotoxic for activated T cells (*12–14*). More than 60 patients have received this fusion toxin in phase I and II clinical protocols. DAB_{486} IL-2 was well tolerated. The maximum tolerated dose (MTD) was that dose at which 30% of the patients exhibited asymptomatic hepatic transaminase elevations. Antitumor effects have been seen in approximately 40% of patients; responses have been noted in B cell leukemias and lymphomas, cutaneous T cell lymphoma, and Hodgkin's disease (*15, 16*).

We tested the impact of DAB_{486} IL-2 treatment on the viability of HIV-1–infected T cells. The viability of uninfected, resting T cells was not reduced by incubation with up to 10^{-7} M DAB_{486} IL-2. These results confirm and extend observations that demonstrated sensitivity of IL-2R–positive T cells to DAB_{486} IL-2 (*11*). Treatment with 10^{-8} M DAB_{486} IL-2 transiently impairs proliferation of uninfected cells in response to 10^{-9} M IL-2 (*17*). HIV-1–infected cells were eliminated by the addition of 10^{-8} M DAB_{486} IL-2, even in the presence of 10^{-9} M IL-2, which is necessary to maintain viability of these T cells in culture (Fig. 1). IL-2 binds to the IL-2R 10- to 100-fold more effectively than DAB_{486} IL-2 (*11*); thus, exogenous IL-2 can compete with DAB_{486} IL-2 for IL-2R occupancy and block DAB_{486} IL-2 activity. Infected cells that were not exposed to DAB_{486} IL-2 were >75% viable after 2 weeks of incubation. These results indicate that the reduction in viability observed in HIV-1–infected cultures exposed to DAB_{486} IL-2 was a specific event and not due to viral replication.

In order to evaluate the selective elimination of HIV-1–infected cells, we mixed uninfected T cells with HIV-1–infected T cells, treated the cultures with DAB_{486} IL-2, and analyzed the cultures for expression of HIV-1 proteins and the production of infectious virus. We incubated $CD4^+$ T cells with HIV-1 for 1 hour to allow infection to occur. Although cell proliferation was transiently inhibited after addition of DAB_{486} IL-2, subsequent proliferation was rapid and the number of viable cells in the fusion toxin–treated group equaled the number in the untreated group by the time of analysis (2 weeks after infection). The production of HIV-1 proteins (gp160, p55, and p24) was not detected by specific immunoprecipitation and subsequent gel analysis of DAB_{486} IL-2–treated cell cultures (Fig. 2). However, as anticipated, normal cellular class I major histocompatibility complex (MHC) proteins were produced in both uninfected and infected T cell cultures (Fig. 2). Because cell viability was >95% in both groups, the cytotoxic action of DAB_{486} IL-2 was probably limited to the HIV-1–infected subset of cells in the mixed cultures. Additional cell cultures were examined for expression of HIV-1 p24 in an enzyme-linked immunosorbent assay (ELISA) after incubation with or without DAB_{486} IL-2. Cell culture supernatant fluid was assayed at various times for the presence of p24 (Table 1). The addition

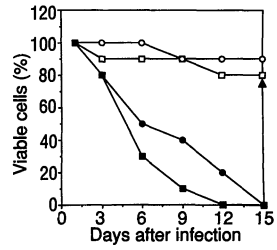

Fig. 1. DAB_{486} IL-2 selectively killed HIV-1–infected T cells. We prepared $CD4^+$ cells (from the peripheral blood of HIV-1–negative blood donors) by negative selection to remove B cells, macrophages, natural killer cells, and $CD8^+$ T cells. B cells and macrophages were removed by passage over nylon wool columns. $CD8^+$ and $CD16^+$ cells were incubated with antibodies to CD8 (OKT8, American Type Culture Collection) and CD16 and then removed with antibody-coated magnetic beads (*26*). Cells (2×10^6 cells/ml) were cultured in 8 ml of RPMI 1640 medium and 10% bovine calf serum (BCS). Cells were infected by incubation with $HIV\text{-}1_{IIIB}$ (derived from filtered supernatants of infected H9 cells, AIDS Research and Reference Reagent Program, Bethesda, Maryland). DAB_{486} IL-2 (10^{-7} or 10^{-8} M) was added on days 1 and 3 after infection. Cells were passaged twice weekly, and viability was determined on the basis of Trypan blue exclusion. Circles represent uninfected (open) and infected (closed) cells treated with 10^{-8} M DAB_{486} IL-2. Squares represent uninfected (open) or infected (closed) cells treated with 10^{-7} M DAB_{486} IL-2. Uninfected cells were >90% viable at 2 weeks; infected cells (without DAB_{486} IL-2) were >75% viable at 2 weeks (closed triangles). Percent viability is representative of three experiments, each with lymphocytes from three different donors.

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Table 1. Incubation of mixed T cell cultures with DAB_{486} IL-2 eliminated production of p24 antigen. Each culture contained a total of 10^6 T cells, prepared as in Fig. 1. Mixed cultures contained 10^7 $CD4^+$ T cells and 10^6 HIV-1–infected cells. Cultures were treated as described in Fig. 2, and supernatants were assayed for p24 in an ELISA assay (Abbott Laboratories) 2 days after resuspension. These data are representative of three experiments performed.

Days in culture	Culture conditions*			
	Uninfected T cells	HIV-1–infected T cells	Mixed T cell cultures	Mixed T cell cultures + DAB_{486} IL-2†
6	0	0	0	0
9	0	256	0	0
12	0	>500	89	0
15	0	>500	298	0
18	0	>500	>500	0

*Picograms of p24 in the supernatant, measured 2 days after cell washing. †DAB_{486} IL-2 (10^{-8} M) was added on days 1 and 3.

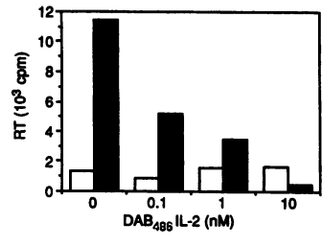

Fig. 3. Addition of DAB_{486} IL-2 eliminated HIV-1 replication in monocytes. Aliquots of counterflow centrifugal elutriation-purified monocytes (*27*) (10^7) were suspended in 1 ml of primary macrophage culture supernatant containing $HIV\text{-}1_{Ba\text{-}L}$ (*28*). Additional aliquots of monocytes were suspended in parallel with RPMI 1640 (Gibco) containing 10% fetal calf serum (control) for 1 hour with intermittent shaking at 37°C. Washed monocytes were plated and DAB_{486} IL-2 (10^{-8} to 10^{-11} M) was added, beginning on day 3 [when IL-2R first appeared in infected monocytes (*4*)] and every third day thereafter. Supernatants from the infected (shaded bars) and control (clear bars) adherent monocyte cultures were assayed directly for RT activity (day 20 shown) by a micromethod (*19, 28*) modified from Spira and co-workers (*29*). Each point represents the average of six determinations. This experiment is representative of five performed.

of DAB_{486} IL-2 to mixed T cell cultures prevented expression of p24 antigen.

To further assess the elimination of infectious virus from mixed cell populations, we cocultivated DAB_{486} IL-2–treated or untreated cultures with the H9 tumor cell line. The virus was produced in the untreated cell cultures at all time points assayed, as measured by p24 antigen in culture supernatants (Table 2). However, infectious virus could not be detected by cocultivation in mixed cell cultures that were exposed to DAB_{486} IL-2 (DAB_{486} IL-2 added on days 1 and 3) when cells were removed on days 6 and 9. Thus, a loss of infectious virus was achieved by 3 days after the second addition of DAB_{486} IL-2 (*18*). Thus, the fusion toxin, DAB_{486} IL-2, can be used to abrogate expression of viral proteins and to selectively eliminate HIV-1–infected T cells from mixtures of infected and uninfected T cells.

Because macrophages serve as a reservoir for HIV-1 (*19*), the effect of DAB_{486} IL-2 on infected macrophages was examined. Cultures of peripheral blood monocytes were infected with a macrophage tropic virus ($HIV\text{-}1_{Ba\text{-}L}$) and then treated with varying doses of DAB_{486} IL-2, beginning on day 3 after infection. Researchers initiated treatment with DAB_{486} IL-2 when IL-2Rs began to emerge on infected cells (*4*) and before evidence of productive replication of the virus. Incubation of infected cells with 10^{-8} M DAB_{486} IL-2 eliminated viral transcription [assessed by supernatant levels of viral reverse transcriptase (RT) activity, Fig. 3]. Lower concentrations of the fusion toxin inhibited RT to a lesser extent. Adherent monocytes and macrophages remaining in the DAB_{486} IL-2–treated cultures were viable and morphologically indistinguishable from the untreated cell populations.

Because viral infection often leads to expression of both virus-specific and host-encoded proteins on the surface of infected cells, the use of specifically targeted toxins is a logical method for eliminating HIV-1–infected cells without harming the host. A CD4-*Pseudomonas* exotoxin fusion protein will kill HIV-1–infected cells (*20*), and gp120-expressing tumor cells can be killed with a CD4–ricin A conjugate (*21*). Both of these approaches require active replication of virus and gp120 expression on the cell surface and use CD4 as the targeting agent. The affinity of CD4 for cell surface gp120 varies widely between laboratory and clinical isolates of HIV-1, with clinical isolates resistant to soluble CD4 (*22*). In contrast, because the appearance of IL-2Rs is associated with activation events that do not require expression of viral proteins on the cell surface (*1, 3, 4*), DAB_{486} IL-2 may selectively eliminate HIV-infected cells before viral replication.

Quiescent and activated T cells may bind and internalize virus, but HIV-1 in quiescent cells is labile and spontaneously disappears if virus-bearing cells are not activated (*7*). Because infection by HIV-1 does not lead to expression of the IL-2R on all T cells (*23*), the prevention of expression of viral proteins may be related to elimination of activated cells that selectively replicate the virus. The virus appears to be labile in quiescent T cells and may disappear without replication (*7*). Observations suggest that activated B cells may enhance HIV-1 replication in T cells and monocytes through the release of cytokines (*8*). Thus, regardless of the mechanism involved in cell activation, the therapeutic use of a molecule that selectively eliminates activated cells might be able

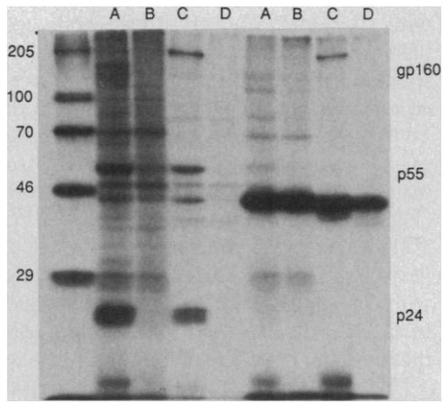

Fig. 2. DAB_{486} IL-2–treated T cell cultures did not produce HIV-1 proteins. T cells, infected as described in Fig. 1, were incubated overnight in 10% BCS and washed to eliminate free virus three times before addition to uninfected T cells from the same donor. One million infected T cells were mixed with 10^7 uninfected T cells and cultured in RPMI 1640 and 10% BCS supplemented with Lymphocyt T, 10 units per milliliter, or approximately 10^{-9} M IL-2 (Lymphocult T, Boehringer Mannheim), with or without DAB_{486} IL-2 (10^{-8} M) at 10^6 cells/ml in six-well cluster dishes. Cells were pelleted, washed twice, and resuspended every 2 days in IL-2–containing medium. DAB_{486} IL-2 was added on days 1 and 3 and washed out 24 hours later. Uninfected cell cultures were treated in an identical manner. Two weeks after infection, cells were labeled overnight with [^{35}S]methionine (25 μCi/ml). The labeled cells were lysed and immunoprecipitation was performed with antibody to HIV (AIDS Research and Reference Reagent Program) (left lanes A through D) or with the W6/32 antibody (American Type Culture Collection) to the monomorphic determinants on the human leukocyte antigen A, B, and C molecules (right lanes A through D). Immunoprecipitated proteins were separated on an SDS–polyacrylamide gel (10%). Lane A, infected T cells; lane B, uninfected T cells; lane C, 10^6 infected cells mixed with 10^7 uninfected cells; lane D, identical to lane C, but 10^{-8} M DAB_{486} IL-2 was added on days 1 and 3 after infection. Positions of molecular size standards (in kilodaltons) are indicated at left margin; positions of HIV-1 proteins are indicated at right margin.

to reduce the amount of virus in HIV-1–infected individuals.

Serum concentrations of DAB_{486} IL-2 used in the experiments described here (10^{-8} M) have been safely achieved in patients with IL-2R–expressing malignancies (*16*). Binding and internalization of DAB_{486} IL-2 by high-affinity IL-2R–bearing cells occurs within 30 min of exposure, resulting in maximal protein synthesis inhibition within 4 to 6 hours and cytotoxicity within 48 to 72 hours (*24*). Therefore, despite the short serum half-life of DAB_{486} IL-2 (5 to 10 min), continuous administration of DAB_{486} IL-2 to patients should not be necessary.

Antitumor effects have been observed in refractory leukemia-lymphoma patients, and these effects have occurred despite the presence of antibodies to diphtheria toxin in some patients, and the presence of elevated soluble IL-2R levels in all patients. This observation is consistent with data obtained by ourselves and others that suggest that soluble IL-2Rs do not interfere with binding of IL-2 to the high-affinity IL-2R (*25*). Extensive immunological monitoring has been an important feature of early clinical trials, and patient data have demonstrated that DAB_{486} IL-2 has no generalized immunosuppressive properties (*15, 16*). The experimental results reported here indicate that DAB_{486} IL-2 selectively eliminates HIV-1–infected cells from mixed T cell cultures while sparing uninfected cells. In addition, DAB_{486} IL-2 inhibits production of viral proteins and infectious virus in T cells and inhibits viral RT in HIV-infected monocytes. Thus, the emergence of IL-2Rs on monocytes and lymphocytes that are replicating HIV-1 may provide the basis for early therapeutic intervention with targeted toxins in HIV infection.

Table 2. Incubation of T cell cultures with DAB_{486} IL-2–eliminated infectious virus. $CD4^+$ T cells were infected with HIV-1, washed, and then mixed with uninfected cells 1 day later, as described in Table 1. Mixtures (uninfected:infected T cells) are a ratio of 10:1. Cells were cultured in IL-2 (10^{-9} M), with and without DAB_{486} IL-2 (10^{-8} M) added on days 1 and 3. Cultures were washed and fed every 48 hours as described in Table 1. At several time points, T cells (5×10^5) were removed, washed three times, and cocultivated with 5×10^5 uninfected H9 cells. Cultures were incubated for an additional 6 days and screened for production of p24 in the supernatants. These data are representative of two experiments, each with duplicate culture wells.

T cell cultures	DAB_{486} IL-2	Days after infection	p24 (pg)
Uninfected		0	
HIV-1–infected		0	>500
Mixtures		0	>500
Mixtures	10^{-8} M	0	>500
Mixtures		1	>500
Mixtures	10^{-8} M	1	>500
Mixtures		6	>500
Mixtures	10^{-8} M	6	0
Mixtures		9	>500
Mixtures	10^{-8} M	9	0

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Requirement of Heparan Sulfate for bFGF-Mediated Fibroblast Growth and Myoblast Differentiation

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Basic fibroblast growth factor (bFGF) binds to heparan sulfate proteoglycans at the cell surface and to receptors with tyrosine kinase activity. Prevention of binding between cell surface heparan sulfate and bFGF (i) substantially reduces binding of fibroblast growth factor to its cell-surface receptors, (ii) blocks the ability of bFGF to support the growth of Swiss 3T3 fibroblasts, and (iii) induces terminal differentiation of MM14 skeletal muscle cells, which is normally repressed by fibroblast growth factor. These results indicate that cell surface heparan sulfate is directly involved in bFGF cell signaling.

BASIC FGF IS A PROTOTYPE OF THE heparin-binding growth factor (HBGF) family that includes acidic fibroblast growth factor (aFGF), int-2, *hst*, FGF-5, k-FGF, and KGF, polypeptides that control cell growth and differentiation (*1*). HBGFs bind to heparan sulfate and to high-affinity protein receptors on cell surfaces (*2*). Cross-linking of iodinated bFGF to cell surfaces identifies multiple high-affinity receptors that bind the growth factor with dissociation constants in the 10^{-9} to 10^{-12} M range (*1, 3*). Among these are the *flg* and *bek* gene products, transmembrane proteins that contain cytoplasmic domains with tyrosine kinase activity (*4*). FGF binding triggers intracellular phosphorylation of several proteins, including the receptors themselves, which may lead to FGF action (*5*).

Heparan sulfate binds bFGF with a dissociation constant of 10^{-8} to 10^{-9} M and is present on proteoglycans found in the extracellular matrix or anchored in the plasma membrane (*2, 6, 7*). An example of the latter is syndecan (*8*). Syndecan derived from several cell types, including NMuMG mammary epithelial cells, Swiss 3T3 cells, or MM14 myoblasts, binds to bFGF affinity columns (*9, 10*), and expression of syndecan in lymphoblastoid cells confers FGF binding on these cells (*11*). Indirect roles for heparan sulfate in bFGF activity include extracellular stabilization and storage of FGF (*12, 13*). A direct role in bFGF activity has not been apparent, although deletions of putative heparin or heparan sulfate binding regions

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