RAPID COMMUNICATION

Targeting of Human Immunodeficiency Virus-Infected Cells by CD8+ T Lymphocytes Armed With Universal T-Cell Receptors

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We have developed an immunotherapeutic approach with potential application in the treatment of viral and malignant disease. We show that primary CD8+ T cells isolated from peripheral blood can be genetically modified by retroviral transduction to express high levels of universal (major histocompatibility complex-unrestricted) chimeric T-cell receptors specific for human immunodeficiency virus (HIV) antigens. Two classes of HIV-specific URs in which the antigen-binding domain is comprised of either CD4 or a single-chain antibody are capable of activating a number of T-cell effector functions in response to target cells, including cytolysis, in a highly sensitive and specific manner. Importantly, we have addressed a number of issues which, although particularly relevant to the clinical application of this approach in the treatment of HIV infection, may also impact on the potential of UR immunotherapy for other disease targets. The UR immunotherapeutic system is particularly suited for evaluation in the clinical setting.

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THE CELL-MEDIATED immune response plays an essential role in the host's defense against viral infection. Studies of cytomegalovirus (CMV) and influenza virus for which small animal models are available have shown that CD8+ cytotoxic T lymphocytes (CTLs) represent the major component of this cellular immunity. The therapeutic efficacy of adoptive T-cell transfer as a treatment for viral disease has been demonstrated in animal models and is under evaluation in human studies. The potential of this approach in the clinical treatment of viral infection was highlighted by a recent study in which CMV-specific CD8+ CTL clones isolated from bone marrow (BM) donors were shown to restore viral immunity upon adoptive transfer to immunodeficient BM transplant recipients.

Although optimal animal models for human immunodeficiency virus (HIV) infection await development, evidence that CD8+ CTLs represent the major and earliest immune response to HIV infection is supported by correlative data from HIV-infected patients. Such studies have shown that HIV-specific CTL are present in the peripheral blood mononuclear cells (PBMCs) of HIV seronegative individuals exposed to HIV. Although HIV antigen-specific CD8+ T cells are also found at high frequency in the peripheral blood of asymptomatic HIV seropositive patients, a selective decline in HIV-specific CTL levels is observed in patients as they progress to later stages of the disease. Therefore, the clinical data available suggest that a breakdown of the host cell-mediated immune response may be responsible for progression to symptomatic acquired immunodeficiency syndrome (AIDS). In vitro studies have not only confirmed that HIV-specific CD8+ T cells exhibit cytolytic activity toward HIV-infected targets, but have also shown that CD8+ T cells have the ability to inhibit replication of HIV in lymphocyte cultures. Data supportive of the central role of CD8+ T cells in HIV infection suggest that adoptive transfer of HIV-specific CD8+ T cells may have potential as an immunotherapy for HIV-infected individuals.

Current approaches to adoptive immunotherapy for viral disease require the isolation and expansion in vitro of major histocompatibility complex (MHC)-restricted T cells with appropriate antigenic specificity. This process can be both laborious and lengthy, requiring isolation of MHC-matched viral-specific T cells and antigen-presenting cells from virally infected individuals, followed by subsequent in vitro expansion of MHC-restricted T cells over a period of months to attain the large cell numbers required for therapeutic application. Furthermore, the MHC-restricted nature of viral antigen recognition by the T-cell receptor limits the application of this approach to MHC-matched individuals. An ideal strategy for the treatment of viral infections would provide a rapid and efficient means of generating large numbers of viral antigen-specific effector cells from selected lymphocyte populations that possess effector functions with potential therapeutic activity. Furthermore, the system should be sufficiently flexible to allow not only preselection of effector cell type, but also effector cell recognition of viral antigen(s) of choice and potential targeting of multiple antigens. Finally, the system should be directly applicable to the clinical setting, and ultimately have broad potential in the treatment of MHC-unmatched individuals.

In this report, we describe a strategy for adoptive immunotherapy of viraly infected individuals that addresses the issues identified above and overcomes some of the inherent limitations of current approaches. Using HIV as a model system, we have developed a strategy in which universal (MHC-unrestricted) chimeric T-cell receptors (URs) are used to redirect the antigenic-specificity of primary CD8+ T-cell populations with potential therapeutic activity to recognize viral antigen(s) of choice expressed on the surface of infected cells. Upon binding to viral antigen, these URs initiate T-cell activation, resulting in induction of effector functions, including cytolysis of the virally infected cell.

We and others have exploited the ability of the cytoplasmic domain of the invariant ζ chain of the T-cell receptor
to endow certain heterologous receptors with the ability to stimulate activation responses, including lymphokine production and cytolytic function. Specifically, it has been shown that chimeric molecules in which the interleukin-2 (IL-2) receptor α chain or the CD8 receptor are fused to ζ initiate cytokine secretion in response to cross-linking by antibodies when expressed in transformed mouse and human T-cell lines, respectively.19,20 Furthermore, CD4- and CD16-ζ receptors expressed transiently at high level in CTL clones by vaccinia vectors have been shown to initiate cytolytic activity against target cells expressing vaccinia-HIV env or anti-CD16 antibodies, respectively.21,22 In a later study, it was shown that a chimeric ζ receptor in which the antigen-binding domain was derived from an antibody specific for the hapten trinitrophenyl (TNP) was able to initiate killing of a target cell line chemically modified with TNP, when expressed in a mouse hybridoma cell.23 The functional studies with ζ chimeric receptors described above have been limited to transformed mouse or human cell lines into which genes can be readily transferred19,20,23 or to CTL clones using transient vaccinia-based systems that result in host cell death.21,22

We have designed two different classes of HIV-specific URs in which the cytoplasmic domain of ζ is fused to an extracellular domain which confers specificity for the envelope (env) glycoprotein of HIV-1. HIV env is the major immunogenic protein encoded by the virus, and is synthesized as a 160-kD glycosylated precursor, gp160, on the rough endoplasmic reticulum of infected cells.24 HIV env subsequently undergoes intracellular cleavage to produce an external glycoprotein of 120 kD, gp120, and a 41-kD glycoprotein, gp41, anchored in the cell by a transmembrane domain with which it is noncovalently associated.25,27 The first class of UR derives its specificity from the extracellular domain of the human CD4 receptor that recognizes the gp120 moiety of HIV env, currently the only well-characterized high-affinity receptor for HIV.28,29 For the second class of UR, the extracellular domain is derived from a human antibody specific for the gp41 moiety of HIV env30,31 and consists of a single chimeric molecule bearing a functional antigen binding domain in which the variable domains of the Ig heavy and light chain genes are covalently linked via a peptide tether.

Initial functional studies in which both the CD4-ζ and single-chain antibody (SAb)-ζ URs were evaluated in a model T-cell line showed their ability to initiate T-cell effector functions such as cytokine secretion in response to target cells expressing low levels of HIV env. We subsequently focused our efforts toward developing an experimental system with which we could evaluate the ability of CD4- and SAb-URs to redirect PBMC-derived CD8+ T cells toward a virally infected cell. Because lymphocytes are highly refractory to conventional gene transfer and expression methods, a high-efficiency gene transfer and expression system for primary CD8+ T cells compatible with clinical applications such as adoptive immunotherapy was essential. Approaches that rely on retroviroirally mediated transduction have been limited by the relatively poor expression in lymphocytes achieved compared with other cell types.25 For example, tumor-infiltrating lymphocytes (TILs) retrovirally transduced with a tumor antigen-specific chimeric receptor exhibit very poor cytolytic activity against a target tumor cell line.25 The chimeric receptor used in this study possessed the signal transducing subunit of the Fc receptor γ chain (FcRγ), a protein with similar structure and function to the ζ TCR chain.26 The poor cytolytic activity exhibited by the chimeric FcRγ-transduced TILs described in this report may be related to the low levels of gene expression obtained with the retroviral vector system used in this study,27 and places a serious limitation on the development of this immunotherapeutic approach.

In contrast to the studies described above, we have succeeded in obtaining genetically modified primary human CD8+ T cells stably expressing high levels of HIV-specific URs by retrovirally mediated transduction. This report describes, for the first time, an immunotherapeutic approach involving chimeric receptor-redirecded T cells, which can be directly evaluated in the clinical setting. We show that CD4- and SAb-URs expressed in PBMC-derived CD8+ T cells mediate highly efficient proliferation and cytolytic function in response to target cells expressing very low levels of HIV antigen, thereby confirming their functional activity and sensitivity. Importantly, we show that both classes of UR+ CD8+ T cells exhibit highly efficient cytolytic activity against CD4+ T cells infected with HIV, in an antigen-specific MHC-unrestricted manner. Furthermore, we have addressed a number of issues that are pertinent to the clinical relevance of the UR approach in the treatment of viral infection. We show that HIV-specific antibodies and free virus/antigen do not inhibit the cytolytic activity of UR+ T cells, and also that UR+ T cells generated upon antigenic stimulation via the UR retain cytolytic activity. The ability to redirect effector T-cell populations with desirable therapeutic properties to recognize target cells of choice makes this a potentially useful immunotherapeutic approach not just for the treatment of HIV infection, but also for other viral and malignant diseases.

MATERIALS AND METHODS

HIV-specific CD4- and SAb-URs. Both URs possess the transmembrane (TM) domain of CD4 (residues 372 to 395 of the mature CD4 chain), and the cytoplasmic domain (CYT) of ζ (residues 31 to 142 of the mature ζ chain). The extracellular domain (EC) of the CD4-UR consists of all four Ig-like (V1-V4) domains of the CD4 receptor (residues 1 to 372 of the mature CD4 protein). The EC domain (SAb) of the SAb-UR receptor was constructed from the gp41-specific human monoclonal antibody (MoAb) 98.6,30,31 The antigen-binding domain consists of a single-chain antibody variable fragment (scFv) in which residues 1 to 107 of the variable region (VL) from the mature light chain gene of MoAb 98.6 are linked directly to amino acids 1 to 113 of the mature 98.6 MoAb heavy-chain variable domain (VH) via a 14-amino acid linker. The VL-VH antigen-binding domain is fused to the IgGl heavy-chain constant region at amino acid 234. Disulfide bonds are indicated by S-S. The heavy chain and κ light-chain cDNAs were generated by reverse transcription of RNA isolated from the cell line SP-1/98.6.30,31 Oligonucleotide-directed deletional mutagenesis was used to form specific junctions between gene sequences.35

Generation of target cells expressing HIV env: CEM/IIIB and 293env. The CD4+ T-cell line, CEM, was infected with the viral isolate HIV-1 IIIB as previously described.36 Additional target cell lines expressing both gp120 and gp41 were generated from the hu-
man cell line 293T by coelectroporation of pKl1.neo and pCMVenv. pKl1.neo confers resistance to G418 (King L, Finer M.H., unpublished data, June 1991) and pCMVenv encodes rev and env (gp160) from the HXB2 HIV-1 clone, under the control of the simian CMV immediate early promoter. G418-resistant clones were isolated and analyzed for expression of the env proteins gp120 and gp160 by immunoblotting with an anti-gp120 MoAb 9305 (NEA). 293c cells were resuspended at 1 x 10^6 cells/10 mL of NP40 lysis buffer (1% NP40, 150 mMol/L NaCl, 50 mMol/L Tris [pH 7.8], 0.1% sodium dodecyl sulfate [SDS], 10 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mMol/L phenylmethylsulfonyl fluoride, and 0.1 mol/L iodoacetamide), and incubated at 4°C for 30 minutes; the lysate was centrifuged at 12,000g and fractionated by 4% to 16% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose (Amer sham, Arlington Heights, IL) and filters incubated with an anti-gp120 MoAb 9305 (NEA). Bound antibody was detected with horseradish peroxidase-conjugated antibody to mouse IgG, followed by a nonisotopic enhanced chemiluminescence ECL assay (Amersham). Two representative clones, 293v1193 and 293v18, were selected for use in studies for evaluation of UR function. The 293neo clone contains the pKl1.neo plasmid only, and was used as a negative control.

Surface expression of gp120 and gp41 in CEM T lymphoblastoid cells infected with HIV-1 and in 293 cells stably expressing gp120 (293env13) was determined by flow cytometry with a gp120-specific MoAb F105 or with a soluble Sab derived from the extracellular domain of the gp41-specific SAb-UR. Cells were first stained with the unlabeled MoAb or SAb, followed by fluorescein isothiocyanate (FITC)-conjugated goat-antihuman IgG antibody, and then analyzed by flow cytometry.

Generation of Jurkat cells stably expressing HIV-URs. Constructs encoding CD4-UR, Sab-UR, and native CD4 (nCD4) were introduced into a mammalian expression vector bearing a selectable marker, pKl1.RSV-neo (M.H.F., T.J.D., unpublished data, June 1991). Each construct was introduced into the human T-helper cell line Jurkat by electroporation, and independent clones were subsequently obtained by limiting dilution and selection in G418. Individual clones expressing high surface levels of the introduced gene were identified by FACS analysis with fluorescent antibodies specific for either CD4 or the Fc domain of human IgG. Although Jurkat cells express a low level of native CD4 on the cell surface, expression of CD4-UR or high levels of nCD4 was readily detected above background levels, and subsequently could be confirmed by immunoprecipitation (L.Q., M.R.R., unpublished data, August 1991).

IL-2 assays. Jurkat clones expressing either CD4-UR, Sab-UR, or nCD4 were evaluated for their ability to secrete IL-2 in response to incubation with the two target cell lines, 293env13 and 293env18, and the 293neo control. Jurkat clones were incubated with 293 target cells at a ratio of 1:1 in a 96-well plate for 18 to 24 hours in the presence of 5 ng/mL phorbol myristate acetate (PMA). In parallel, Jurkat clones were stimulated via the native T-cell receptor by crosslinking with immobilized MoAb OKT3 (Ortho, Raritan, NJ) and PMA (5 ng/mL), or were activated by incubation with the calcium ionophore ionomycin (IONO) at 1 μmol/L and PMA (5 ng/mL). Duplicate wells were set up for each experimental condition. Supernatants were removed and assayed for IL-2 concentration (picograms per milliliter) by solid-phase ELISA (R and D Systems, Minneapolis, MN). Results obtained upon incubation of the cells with the MHC class I-specific antibody, W6/32, were similar to those obtained in media plus PMA alone.

Retroviral transduction and phenotypic analysis of human PBMC-derived CD8+ T cells. PBMCs were purified from human blood by Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation, washed, resuspended at 5 x 10^6 cells in 4 mL phosphate-buffered saline (PBS) containing 0.5% human gamma globulins. After a 1-hour incubation of the PBMC suspension in anti-CD8 antibody-coated T-25 tissue culture flasks (Applied Immune Sciences, Santa Clara, CA), CD8+ T cells were simultaneously retracted and activated by incubation in T-cell medium (10% fetal calf serum, RPMI 1640, 1% nonessential amino acids, 10 mMol/L HEPES buffer, 2 mMol/L glutamine, 25 μMol/L 2-mercaptoethanol) containing 10 ng/mL OKT3 (Ortho) and 64 U/mL IL-2 (Pharmacia, Piscataway, NJ) for 48 hours. Where necessary, residual cells crossreacting with either the CD4-specific antibody used to detect CD4-UR surface expression, or the human Fc-specific antibody used to detect Sab-UR expression, were removed from the enriched CD8+ population by negative panning using tissue culture flasks coated with the appropriate antibody. Purified cells were finally washed and allowed to recover in fresh T-cell medium plus IL-2 for 24 hours before transduction. CD8+ T cells prepared in this manner were usually greater than 95% CD8+ and CD3-, and less than 0.5% CD4+ or Fc-.

Highly efficient gene transfer of HIV-specific URs into the purified CD8+ T-cell population was performed by using the novel packaging system designated kar to produce high-titer recombinant retrovirus. Transduced CD8+ T cells expressing surface CD4-UR or Sab-UR were subsequently identified by flow cytometry and purified by positive panning with tissue culture flasks coated with either anti-CD4 or antihuman Fc MoAbs, respectively. T-cell populations were maintained by periodic stimulation with OKT3 at 10 ng/mL or with immobilized OKT3, in the presence of allogeneic mitomycin C-treated PBMCs. Genetically modified and unmodified CD8+ T-cell populations derived from the clones described above were dually labeled with phycoerythrin (PE)-conjugated anti-CD8 MoAb and either FITC-conjugated anti-CD4, antihuman Fc MoAb, or anti-CDS to detect expression of CD4-UR, Sab-UR, or native TCR/CD3, respectively. Cells were analyzed for double-color immunofluorescence in a FACScan cytometer (Becton Dickinson, San Jose, CA). Quadrants were set by labeling each T-cell population with FITC- and PE-conjugated isotype-matched control antibodies.

Proliferation assays. Unmodified, CD4- or Sab-UR expressing PBMC-derived CD8+ T cells were incubated at 1 x 10^6 cells/well with equal numbers of mitomycin C-treated 293neo or 293env13 cells (cells were treated with 200 μMol/L mitomycin C for 30 minutes at 37°C) in the presence of T-cell medium (described above) plus 64 U/mL IL-2 (Pharmacia). Cells were incubated for 3 days, pulsed with 1 μCi [3H]thymidine overnight, and [3H]thymidine-labeled DNA retained by living cells (spontaneous). All experiments were performed as described above. Evaluation of cytolytic activity in the presence of HIV-specific antibodies and free virus/antigen. Specific lysis was determined by the JAM test as described above. All experiments were performed at an E:T ratio of 1. HIV antigen was obtained directly from the supernatant of an H9 culture chronically infected by HIV-1 MN strain. The concentration of viral antigen was determined by the DuPont HIV-1 Core Profile ELISA assay. The p24 content of the
seropositive serum samples ranged from undetectable to 110 pg/mL with a mean of 13.6 pg/mL.

RESULTS

Structure of anti-HIV URs: CD4-ζ and SAb-ζ. The extracellular domain of the first class of anti-HIV UR, CD4-ζ, is derived from the CD4 receptor as shown in Fig 1. Native ζ exists as a disulfide-linked homodimer or as a heterodimer in association with an alternatively spliced product of the ζ gene, etc., by virtue of a membrane proximal cysteine residue at position 11 of the mature ζ chain.42 However, because the CD4-ζ receptor possesses the transmembrane domain of the native CD4 chain, it is expressed as a monomer (L.Q., M.R.R., unpublished data, August 1991), as is the native CD4 receptor.42 The extracellular domain of the second class of anti-HIV universal T-cell receptor is derived from the human antibody, 98-6, which recognizes a conformational epitope located within the second immunodominant region of gp41.30 The gp41 transmembrane protein of HIV is present at a mean of 13.6 pg/mL.

Fig 1. Structure of HIV-specific CD4- and SAb-URs. Both URs possess the transmembrane (TM) domain of CD4 (residues 372 to 395 of the mature CD4 chain), and the cytoplasmic domain (CYT) of ζ (residues 31 to 142 of the mature ζ chain). The EC of the CD4-UR consists of all four Ig-like (V1-V4) domains of the CD4 receptor (residues 1 to 372 of the mature CD4 protein). The EC domain (SAb) of the SAb-UR receptor was constructed from the gp41-specific human MoAb 98.6.30,31

98.6, in which an scFv fragment comprising the variable domains of the κ light-chain and heavy-chain genes tethered by a peptide linker is fused to the constant domain of the heavy chain. Joining of the SAb extracellular domain to the cytoplasmic domain of ζ results in formation of the second class of HIV-specific UR, SAb-ζ. The transmembrane domain of both the CD4-UR and SAb-UR is derived from CD4. However, in contrast to CD4-UR, the SAb-ζ receptor is expressed as a disulfide-linked dimer, by virtue of the intermolecular disulfide bonds present in the Fc-hinge domain of the antibody (L.Q., M.R.R., unpublished data, August 1992).

Cytokine secretion in response to HIV env target cells. We initially used the CD4+ human T-helper cell line Jurkat (JK) to screen the two classes of anti-HIV receptors for their ability to initiate T-cell activation in response to viral antigen. Jurkat cells are well characterized with respect to signal transduction, and retain a number of the functional characteristics associated with normal resting human CD4+ T-helper cells, such as the ability to secrete IL-2 upon stimulation of the native T-cell receptor.46 A number of studies suggest that T-helper cell subsets exhibiting a type 1 response, namely production of IL-2 upon activation, may be important in initiating the protective cell-mediated immune responses associated with control of HIV infection.46-48 To evaluate the ability of the URs to mediate T-cell effector functions such as IL-2 secretion in response to interaction with target antigen, suitable target cell lines expressing HIV env were generated from the human embryonic kidney cell line, 293.37 Although surface expression of gp120 and gp41 was not high enough to detect by flow cytometry, cytoplasmic expression of HIV env was confirmed by immunoblotting (Fig 2A). Individual JK clones were identified that stably expressed either CD4- or SAb-UR (L.Q., M.R.R., unpublished data, August 1992), and were evaluated for their ability to secrete IL-2 in response to co-incubation with two independent 293env target cell lines (Fig 2B). These data show that upon interaction with membrane-bound gp120 or gp41, both the CD4- and SAb-UR, respectively, can activate JK cells to secrete IL-2 at high levels. Specifically, the IL-2 response obtained on stimulation of the URs by the 293env cell lines is similar to that obtained on stimulation of the native T-cell receptor using the anti-CD3 MoAb OKT3 as agonist. Jurkat clones expressing high levels of native CD4 were used as negative controls, and do not respond to stimulation by the 293env target cell lines, although their anti-CD3 response is intact (Fig 2B).

Introduction and expression of URs in PBMC-derived CD8+ T cells. Although these initial experiments served to show functionality of the anti-HIV URs in T-cell activation, the subsequent challenge was to evaluate their ability to mediate T-cell effector functions in primary human CD8+ T cells. Therefore, we introduced the URs via retroviral transduction into human CD8+ T cells purified by positive selection from the PBMCs of normal donors (Fig 3). Transduced cells expressing the UR were readily detectable post-transduction by flow cytometry (data not shown), and therefore direct purification of this genetically modified population could be achieved by exploiting surface expression of the CD4- or SAb-UR (Fig 3). Purified populations
of transduced CD8⁺ T cells obtained in this manner were subsequently analyzed by flow cytometric analysis, and high levels of surface expression of the URs was confirmed (Fig 3A). Surface expression of the native T-cell receptor (CD3) in the CD4-UR and SAb-UR CD8⁺ T-cell populations was found to be similar to that of the untransduced T-cell population (Fig 3B). The presence of integrated provirus in the genome of the UR-CD8⁺ T-cell populations was subsequently confirmed by Southern analysis (Fig 4, A and B). The high levels of UR expression observed in this system abrogate the need for the lengthy posttransduction selection period necessitated by other retroviral transfer systems in which expression of a drug-resistance marker such as neo is required⁷⁻¹¹ and also avoid some of the adverse effects that selectable marker expression has been shown to have on long-term gene expression. To date, we have not observed any significant decrease in levels of UR expression in vitro during a 2-month posttransduction period in which genetically modified CD8⁺ T-cell populations generated as described above were analyzed by flow cytometry (L.Q., M.R.R., unpublished data, June 1994).

Proliferation of UR⁺ CD8⁺ T cells in response to HIV env. UR⁺ CD8⁺ T-cell populations were evaluated for their ability to proliferate in response to 293 target cells expressing HIV env. Genetically modified UR⁺ CD8⁺ T cells were incubated mitomycin C-treated 293env cells or control 293neo cells for 3 days, and ³H-TdR incorporation was measured during 18 hours of culture. T-cell populations stimulated with the CD3-specific MoAb OKT3 served as internal controls by providing proliferation responses obtained by efficient cross-linking of the native T-cell receptor. As shown in Fig 5, both CD4-UR⁺ and SAb-UR⁺ CD8⁺ T cells proliferate extensively in response to stimulation by gp120 or gp41 expressed on the surface of 293env cells. Whereas unmodified CD8⁺ T cells do not proliferate in response to 293env cells, the proliferation response of UR-modified T cells from the same donor is similar to (SAb-UR) or exceeds (CD4-UR) that obtained by stimulation via the native T-cell receptor. The UR⁺ CD8⁺ T-cell populations do not exhibit a significant proliferative response to the 293neo control cells. We have also observed that UR⁺ T-cell populations may be expanded in vitro by repeated stimulation of the UR with specific antibody or 293env cells (L.Q., M.R.R., unpublished data, June 1994).

Cytolytic activity of UR⁺ CD8⁺ T cells against HIV env targets. The ability of the anti-HIV URs to mediate killing of HIV env-expressing target cells was determined for both CD4-UR and SAb-UR. UR⁺ CD8⁺ or UR⁺ CD8⁺ T cells were evaluated in a cytotoxicity assay against the two target cell lines, 293 env13 and env18, at a range of E:T ratios. The results summarized in Fig 6A show that whereas unmodified CD8⁺ T cells exhibit little or no activity toward 293env cells, UR⁺ CD8⁺ T cells generated from the same donor exhibit maximal specific lysis as high as 50% to 60% over background levels at E:T ratios of 10:1. Both the CD4- and SAb-URs initiate similar levels of cytolytic activity against 293 env target cells in this assay, exhibiting 30% to 40% specific lysis at E:T ratios as low as 1:1.

The relative affinity of the CD4 receptor for polymorphic sites on MHC class II molecules is thought to be very low in the absence of additional stabilizing T-cell receptor (TCR)-MHC interactions. The inability of the CD4-UR modified T-cell population described above to initiate a cytolytic response against uninfected cells expressing MHC class II was subsequently confirmed by cytotoxicity assays em-
TARGETING OF HIV-INFECTED CELLS BY CD8⁺

A. 1) Unmodified 2) CD4-UR anti-CD4 (FITC)
3) Unmodified 4) SAbUR anti-k (FITC)

B. 5) Unmodified 6) CD4-UR anti-CD3 (FITC)
7) SAb-UR

Fig 3. Surface expression of URs (A) and native TCRs (B) in transduced human PBMC-derived CD8⁺ T-cell populations. Genetically modified and unmodified CD8⁺ T-cell populations derived from identical donors and generated as described in Materials and Methods were dually labeled with PE-conjugated anti-CD8 MoAb and either FITC-conjugated anti-CD4, antihuman Fc MoAb, or anti-CD3 to detect expression of CD4-UR, SAb-UR, or native TCR/CD3, respectively. Quadrants were set by labeling each T-cell population with FITC- and PE-conjugated isotype-matched control antibodies.

Figure employs the human B-cell line Raji, which expresses high levels of MHC class II, as a target (L.Q., M.R.R., unpublished data, June 1994).

Cytolytic activity of UR⁺ CD8⁺ T cells against T cells infected with HIV IIIB. Both the CD4- and SAb-URs expressed on the surface of the transduced CD8⁺ T-cell population are able to initiate a highly effective cytotoxic response against the 293env target cells despite the fact that surface levels of env are not sufficient for detection by flow cytometry. Subsequent studies evaluated the effectiveness with which the UR⁺ CD8⁺ T-cell populations were able to mount a cytolytic response against target cells more closely resembling those encountered in an HIV-infected individual, namely HIV-infected CD4⁺ T cells. The CD4⁺ T-cell line CEM is readily infectable by the laboratory viral isolate HIV-1 IIIB. When CEM/IIIB cells were used as targets in a cytotoxicity assay, the results obtained were even more dramatic than those observed previously with the 293env target cells: E:T ratios as low as 0.075:1 resulted in specific lysis of the virally infected population, with maximal lysis occurring at E:T ratios as low as 2.5 to 5.0:1 (Fig 6B). In contrast to the 293env cell lines used in the studies described above, the env proteins gp120 and gp41 are readily detectable on the surface of the HIV-1 IIIB infected CEM population (CEMIIIIB) as determined by flow cytometry employing an anti-gp120 antibody, F105, or a soluble SAb derivative of the anti-gp41 MoAb, 98.6 (Fig 6C). The explanation for the increased activity of the UR⁺ CD8⁺ T cells against CEM/IIIB compared with 293env may simply reflect differences in surface antigen levels observed between the two target cells, but also may involve cell-specific differences between the CEM T cells and 293 epithelial kidney cells.
Fig 4. Transduced human PBMC-derived CD8+ T cells express high levels of HIV-URs. (A) Schematic diagram of the retroviral vector encoding the CD4-UR (pRTD2.2F3) or SAB-UR (pRTD2.2F15). The 5’-long terminal repeat (LTR) of the vector contains the human cytomegalovirus (CMV) immediate-early enhancer-promoter (indicated as a stippled arrow) in place of the Moloney murine sarcoma virus (MMLV) U3. EcoRV digestion of the transfected pRTD2.2F3 or 2F15 construct yields a 4.2-kb or 4.47-kb fragment, respectively. Upon viral integration and duplication of the MMLV U3 into the 5’ LTR, EcoRV digestion yields a 3.6-kb (CD4-UR) or 3.87-kb (SAB-UR) fragment, characteristic of the integrated provirus, and equivalent to a CD4-UR control retroviral vector, pRTD1.2F3, which contains two identical MMLV LTRs. The UR gene product is expressed as a spliced mRNA initiated from the LTR (shown below the integrated provirus). The U3/R, R/U5, and the CMV IE enhancer-promoter/R junctions and the EcoRV sites are shown. LTRs are shown as striped boxes, thick lines indicate viral sequences, and the UR inserts are shown as black arrows. (B) Analysis of DNA from transduced CD8+ T cells. Genomic DNA (10 μg) was isolated from the CD8+ T-cell populations shown in Fig 3: untransduced T cells (lane 1), CD4-UR transduced T cells (lane 2), and SAB-UR transduced T cells (lane 3). DNA samples were digested with EcoRV and electrophoresed on 0.8% agarose gels. Southern hybridization was then performed with a 32P-labeled 603-bp probe isolated from the cDNA. Lanes 4, 5, and 6: 52, 170, and 520 pg of EcoRV-digested pRTD1.2-F3 control plasmid DNA corresponding to 3, 10, and 30 copies per cell, respectively, of integrated provirus encoding CD4-UR (pRTD1.2-F3 is a control plasmid that differs from pRTD2.2F3 in containing two identical MMLV LTRs). Lane 7: 52 pg of EcoRV-digested pRTD2.2F3 plasmid DNA corresponding to 30 copies per cell of transfected plasmid DNA.

Cytolytic activity of UR+ CD8+ T cells stimulated by HIV env-expressing target cells. In the studies described above, we have shown that UR+ CD8+ T cells expanded in vitro by stimulation of the native TCR with specific antibody show high cytolytic activity against antigen-expressing target cell lines. Because UR T cells administered to an HIV-infected individual would be expected to encounter cells expressing target antigens, we sought to evaluate whether HIV-specific UR+ T cells stimulated via the chimeric receptor by exposure to env-expressing target cells would retain functional-ity. Therefore, we compared the cytolytic activity of unmodified, CD4-UR+ and SAB-UR+ T-cell populations that had undergone two successive rounds of stimulation via the UR (with 293env cells), compared with the native TCR (with OKT3 and allogeneic PBMCs). Figure 7 shows that the specific cytolytic activity of CD4-UR+ or SAB-UR+ T-cell populations (against HIV env-expressing target cells) subjected to successive stimulation by 293env cells is comparable with that of cells which have been stimulated with OKT3 and allogeneic PBMCs. These data, together with the data in Fig 5B, show that UR+CD8+ T cells not only have the potential to expand in response to HIV env-expressing cells, but also retain their cytolytic ability against these HIV env target cells after antigen-mediated stimulation.

Secreted HIV antigens or HIV-specific patient antibodies do not alter the cytolytic activity of HIV-specific UR T cells. As UR+ T cells administered to patients may conceivably encounter secreted antigen and/or HIV-specific antibodies potentially capable of blocking HIV envelope antigen interactions, it was of interest to determine whether such exposure would affect the cytolytic activity of UR T cells. We investigated the effect of serum from seropositive and seronegative donors on cytolytic activity at a low E:T ratio of 1:1 where such effects would be expected to be most pronounced. As summarized in Fig 8A, we observed no blocking of cytolytic activity in the presence of 20% serum from 20 different HIV+ donors or 5 HIV-seronegative donors. Indeed, similar experiments with up to 50% serum, the highest concentration tested, did not show any blocking of cytolytic activity (data not shown). Thus, in contrast to antibody-dependent cell-mediated cytotoxicity (ADCC), which can be blocked by physiological levels of IgG, the cytolytic
TARGETING OF HIV-INFECTED CELLS BY CD8⁺

Fig 6. Human PBMC-derived CD8⁺ T cells expressing HIV-specific URs kill (A) 293 cells expressing low levels of HIV env, and (B) CD4⁺ T cells infected with HIV-1. (A) The cell lines 293env13, 293env18, and 293neo (A) or uninfected or HIV-1 IIIB infected CEM cell populations (B) were used as targets in the cytotoxicity assay. Similar qualitative and quantitative results were obtained from three independent experiments. No corrections were made for infection efficiencies. Similar qualitative and quantitative results were obtained from three independent experiments. (C) Surface expression of gp120 and gp41 in (a) CEM T-lymphoblastoid cells infected with HIV-1 and (b) 293 cells stably expressing gp160 (293env13). Cells were analyzed by flow cytometry. The light lines show labeling with a negative control MoAb, the dark lines show labeling with the gp120-specific MoAb F105 or with a soluble SAb derived from the extracellular domain of the gp41-specific SAb-UR shown in Fig 1.

activity of UR T cells is unaffected by normal serum. More importantly, the inability of HIV seropositive serum to block cytolysis indicates that HIV-specific antibodies in serum do not compete quantitatively with URs for gp120 or gp41 on target cells, and that serum does not contain sufficient levels of free HIV antigens to block URs on the effector cells.
infections can be eradicated by adoptive transfer of antigenspecific T cells, together with the findings from clinical studies using adoptive transfer into human subjects, suggests that patients may have to receive cell dosages in excess of $10^9$ antigen-specific lymphocytes to obtain a therapeutic antiviral effect. Therefore, it is essential that effector lymphocyte populations genetically modified to respond to a specific target antigen are able to do so with high specificity and efficiency. Both the CD4-UR and SAb-UR expressing CD8 T-cell populations described here exhibit extremely high cytolytic activities toward virally infected targets, with (500 pg/mL p24gag), to serum from HIV seropositive or seronegative donors had no effect on cytolysis. This experiment demonstrates that both free antigens, and the physiologically relevant mixture of HIV antigen and anti-HIV antibodies, fail to block specific cytolysis. The p24 level of 500 pg/mL selected for this experiment is representative of the relatively high levels observed in some infected individuals late in the course of their disease, and is rarely exceeded.

DISCUSSION

The URs described in this report are capable of recognizing two different HIV antigens present on the surface of an HIV-infected cell, and are able to initiate highly efficient effector T-cell responses including cytokine secretion, proliferation, and cytolytic activity on interaction with target cells expressing surface viral antigen. HIV-specific URs were stably introduced into primary human CD8 T cells by retroviral-mediated transduction, and high expression levels for both the CD4-UR and SAb-UR were observed on the surface of the transduced T-cell population. Extrapolation of the data obtained from animal models in which tumors or viral infections can be eradicated by adoptive transfer of antigenspecific T cells, together with the findings from clinical studies using adoptive transfer into human subjects, suggests that patients may have to receive cell dosages in excess of $10^9$ antigen-specific lymphocytes to obtain a therapeutic antiviral effect. Therefore, it is essential that effector lymphocyte populations genetically modified to respond to a specific target antigen are able to do so with high specificity and efficiency. Both the CD4-UR and SAb-UR expressing CD8 T-cell populations described here exhibit extremely high cytolytic activities toward virally infected targets, with (500 pg/mL p24gag), to serum from HIV seropositive or seronegative donors had no effect on cytolysis. This experiment demonstrates that both free antigens, and the physiologically relevant mixture of HIV antigen and anti-HIV antibodies, fail to block specific cytolysis. The p24 level of 500 pg/mL selected for this experiment is representative of the relatively high levels observed in some infected individuals late in the course of their disease, and is rarely exceeded.
E:T ratios below 0.3:1 sufficient for detection of specific lysis. In a study describing TILs transduced with a tumor antigen-specific Fcγ-based chimeric receptor, demonstration of specific cytolytic function against the tumor target required at least 100-fold higher E:T ratios compared with the HIV-specific CD4- and SAb-URs described above.33 The greater sensitivity of the UR system described here may result from a number of factors, including the high levels of chimeric receptor surface expression obtained, and optimal structure of the ζ-based URs which permits efficient activation. The high surface expression levels observed for the UR system described here may be related to the structure of the chimeric receptor and/or of the retroviral vector used in gene transduction. Studies involving retroviral transduction of exogenous genes in TILs have shown that the vectors used have limited utility because of a transcriptional blockade that impairs expression in lymphocytes compared with nonlymphoid cells.32 In contrast, the retroviral vector we used in this study yields similarly high levels of expression in both lymphocytes and nonimmune cells (M.H.F., T.J.D., unpublished data, June 1993). Furthermore, the retroviral vector does not include a selectable marker such as the neomycin phosphotransferase gene that has been shown to inhibit gene expression in other systems.52,53 The relative contributions made by such parameters as UR structure and cell surface number to overall function are currently under investigation in our laboratory. For example, we have also observed that the origin of the transmembrane domain can significantly impact levels of UR surface expression (M.R.R., L.Q., unpublished data, August 1991).

The UR immunotherapeutic approach described here is particularly suited to evaluation in the clinical setting, and can be used to obtain large populations of antigen redirected T cells in a matter of weeks, in contrast to the lengthy process of selection, characterization, and expansion of lymphocytes with native specificity for target antigens. Furthermore, the ability to preselect the effector lymphocyte cell population for UR-mediated redirection serves to increase the flexibility of this system by expanding the repertoire of cell types whose therapeutic potential may be exploited. For example, CD4+ T-helper cells-type 1 (T(H)1) are categorized by their ability to secrete interferon γ and IL-2, responses associated with induction of protective cell-mediated immune responses in a number of infectious diseases, and also implicated in the control of HIV infection.46-48 The observation that both CD4- and SAb-URs can induce efficient production of IL-2 from a CD4+ T-helper cell line on engagement of surface antigen suggests that adoptive transfer of UR-modified CD4+ cells with a T(H)1 phenotype may have therapeutic potential in the treatment of HIV infection. Similarly, the elaboration of appropriate antibody-URs permits the generation of T-cell populations specific for surface viral antigens of choice, such as those conserved among viral isolates.

We have addressed a number of issues which, although particularly relevant to the clinical application of the UR approach for the treatment of HIV infection, may also impact on the potential of UR immunotherapy for other disease targets. We have shown that UR+ T cells proliferate on exposure to target cells expressing the UR-specific antigen, and importantly, that UR+ T cells generated on antigen stimulation via the UR, retain their cytolytic activity against appropriate target cells in an antigen-specific manner. These data suggest that upon adoptive transfer, UR+ T cells will have the capacity to proliferate in vivo on antigenic stimulation and also retain functionality. Another important issue relevant to general application of UR to the clinical setting is the potential for non-cell-bound antigen (in the form of secreted antigen or free virus) or antigen-specific antibodies to block UR-target cell interactions at levels sufficient to impair cytolytic activity. We have shown that serum from HIV-infected donors does not contain sufficient levels of antibodies or free antigen to inhibit function, and that physiologically relevant mixtures of HIV-specific antibodies and free antigen/virus do not inhibit the cytolytic activity of UR+ T cells against target cells expressing UR-specific antigen. These results suggest that CD8+ T cells directed by universal receptors will retain function under physiologic conditions. Finally, the selection of URs with activity against a wide spectrum of primary HIV isolates will be directly relevant to application of HIV-UR T-cell therapy and is currently under investigation. Ultimately, the therapeutic efficacy and safety of UR+ T-cell immunotherapy for HIV will have to be confirmed in human clinical trials.

One important feature of the URs described here is their ability to recognize antigen in an MHC-unrestricted manner. Although UR T cells may be readily used in an autologous setting, this characteristic may facilitate their application in an allogeneic setting with immunocompromised individuals as recipients, for example. Future applications of this UR-based strategy may also involve the use of "universal donor" T cells with altered immunogenic properties leading to increased tolerance in MHC-mismatched recipients. A modified T cell for therapeutic application that combines the feature of the MHC-unrestricted UR with the ability to be transplanted across MHC barriers may provide a novel approach to the treatment of both viral and malignant diseases in genetically diverse individuals.

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