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Evaluation of Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Cytotoxic T-Lymphocyte Responses Utilizing B-Lymphoblastoid Cell Lines Transduced with the CD4 Gene and Infected with HIV-1

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Analysis of major histocompatibility complex-restricted cytotoxic T lymphocytes (CTL) capable of killing human immunodeficiency virus type 1 (HIV-1)-infected targets is essential for elucidating the basis for HIV-1 disease progression and the potential efficacy of candidate vaccines. The use of primary CD4$^+$ T cells with variable infectivity as targets for such studies has significant limitations, and immortal autologous cells with high levels of CD4 expression that can be consistently infected with HIV-1 would be of much greater utility. Therefore, we transduced Epstein-Barr-virus-transformed B-lymphoblastoid cell lines (LCL) with a retroviral vector, LT4SN, containing the human CD4 gene. Stable LCL in which more than 95% of cells expressed membrane CD4 were obtained. Aliquots were infected with HIV-1, and, after 4 to 7 days, nearly all of the cells contained cytoplasmic *gag* and produced high levels of p24 antigen. The ability of major histocompatibility complex-restricted CD8$^+$ CTL to lyse such HIV-1-infected CD4-transduced LCL (LCL-CD4$_{HIV-1}$) was evaluated. These autologous targets were lysed by CTL generated from an HIV-1-uninfected vaccinee over a broad range of effector-to-target ratios. Similarly, the LCL-CD4$_{HIV-1}$ were efficiently lysed by fresh circulating CTL from HIV-1-infected individuals, as well as by CTL activated by in vitro stimulation. Both HIV-1 *env*- and *gag*-specific CTL effectors lysed LCL-CD4$_{HIV-1}$, consistent with the cellular expression of both HIV-1 genes. The LCL-CD4$_{HIV}$ also functioned as stimulator cells, and thus are capable of amplifying CTL against multiple HIV-1 gene products in HIV-1-infected individuals. The ability to produce HIV-1-susceptible autologous immortalized cell lines that can be employed as target cells should enable a more detailed evaluation of vaccine-induced CTL against both homologous and disparate HIV-1 strains. Furthermore, the use of LCL-CD4$_{HIV-1}$ should facilitate the analysis of the range of HIV-1 gene products recognized by CTL in seropositive persons.

Cytotoxic T lymphocytes (CTL) are essential for host resistance to and control of most viruses, including HIV-1. Thus, evaluation of CTL responses to HIV-1 antigens is important in understanding the pathogenesis of HIV-1 infection and in characterizing the potentially protective immune responses elicited by candidate HIV-1 vaccines (10, 22, 35). Assays to detect HIV-1-specific major histocompatibility complex (MHC)-restricted CTL activity have relied primarily upon the use of recombinant vaccinia viruses to express HIV-1 genes in autologous stimulator and/or target cells (5, 34, 37, 41). This approach has been very informative, but has several limitations. First, in evaluating the HIV-1-specific responses of recipients of vaccines employing recombinant vaccinia viruses, the CTL response to the vaccinia virus vector may interfere with detection of the response to the product of the inserted gene. Second, although CTL that can recognize and lyse target cells expressing abundant amounts of a particular HIV-1 protein can be identified with recombinant vaccinia virus reagents, only inferential assumptions can be made about the efficiency with which such CTL will lyse HIV-1-infected target cells expressing the full range of HIV-1 proteins. Finally, analysis of the relative immunogenicity of distinct viral isolates cannot be easily performed with vaccinia viruses, as it requires cloning the gene from each new isolate of interest and constructing new recombinant vaccinia viruses.

To directly test MHC-restricted CTL responses to HIV-1-infected cells, autologous target cells expressing the CD4 molecule, an accessory molecule that contributes to T-helper-cell activation but also serves as the major cellular receptor for HIV-1 (21, 26), are usually used. CD4$^+$ T cells derived from peripheral blood mononuclear cells (PBMC) can potentially be used as targets for CTL analysis but also have several limitations. First, the CD4$^+$ T-cell population must be activated prior to infection with HIV-1, which can be problematic with the dysfunctional CD4$^+$ population often obtained from HIV-1-infected individuals. Secondly, CD4$^+$ T cells are not immortal and thus usually must be obtained fresh and prepared anew for each assay; this may also be problematic in HIV-1-infected individuals with low CD4$^+$ counts. Finally, the infection rate is variable and unpredictable, and the level of HIV-1 expression may be low, with many cells in the population not expressing HIV-1 genes.

The use of an immortalized, highly infectable, autologous cell would be very useful for these studies. B lymphocytes possess many of the essential qualities for such a cell. B cells

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are readily immortalized with Epstein-Barr virus (EBV) and thus can be expanded and maintained in culture (39). Such EBV-immortalized B-lymphoblastoid cell lines (LCL) have been demonstrated to be efficient in vitro stimulators and targets for CTL responses (33). Additionally, LCL, in which a subset of cells express varying amounts of endogenous CD4, have been shown to be susceptible to HIV-1 infection (6, 7, 9, 25, 31, 38, 40). The ability to infect LCL with HIV-1 has been variable (25, 38) but appears to correlate with the proportion and level of CD4 expression (40). Therefore, we have explored methods to increase CD4 expression in immortalized B cells and have evaluated whether such cells are more susceptible to HIV-1 infection and thus are more suitable stimulator and target cells to examine HIV-1-specific CTL responses.

A highly efficient means for stable introduction of exogenous genes into replicating cells is infection with retroviral particles containing recombinant retroviral vectors (30). LXSN is a retroviral vector which contains the *neo* gene under the control of the Moloney simian virus 40 (SV40) promoter and contains a site for introduction of a gene with expression driven off the Moloney leukemia virus long terminal repeat (29). The complementary DNA encoding human CD4 has been inserted into this site, and infectious viral particles have been produced. In this report we describe the transduction of LCL with the CD4 gene in this retroviral vector and the efficient infection of these cells with HIV-1. CD4-transduced LCL infected with HIV-1 have been tested as stimulator and target cells in CTL assays from both naturally HIV-1-infected individuals and in HIV-1-uninfected persons who have been immunized with candidate HIV-1 vaccines. Our findings indicate that immortalized B cells with stable expression of CD4 can be generated by transduction with the CD4 gene, that such cells are highly susceptible to HIV-1 infection, and that HIV-1-infected LCL may have broad utility in the analysis of CTL responses.

MATERIALS AND METHODS

Study population. Heparinized blood was obtained either from healthy unimmunized HIV-1-seronegative individuals as controls; from initially seronegative or consenting subjects participating in National Institutes of Health AIDS Vaccine Evaluation Group (AVEG) Protocol 002, which includes one to two immunizations with a recombinant vaccinia virus expressing the HIV-1$_{LAI}$ envelope glycoprotein gp160 (Bristol-Myers Squibb), followed by one to two booster injections with a recombinant HIV-1$_{LAI}$ gp160 subunit protein vaccine (MicroGeneSys) (13); or from asymptomatic HIV-1-seropositive participants in a blinded-controlled trial (AVEG Protocol 103) examining augmentation of immunity with a recombinant envelope subunit vaccine, ENV 2-3 (Biocine).

CD4 transduction and HIV-1 infection of LCL. The Moloney virus-based retroviral vector containing the human CD4 gene, pLT4SN, was constructed by insertion into pLXSN of an *Eco*RI-*Bam*HI fragment from plasmid T4-pMV7 (26), kindly provided by D. Litman, containing the full open reading frame for CD4 (29). The resultant vector contains the CD4 gene under the control of the 5′ long terminal repeat and the neomycin phosphotransferase Neo^r gene, for positive selection of transduced cells, under the control of the internal SV40 promoter. The vector was transfected into the amphotropic packaging line PA317, and high-titer virus stocks were obtained by growing the packaging line with the inserted plasmid to 80 to 90% confluence and replacing the drug selection medium with a minimal amount of growth medium for 24 h (28, 29). The absence of helper virus in the producer line was confirmed by the S^+L^- assay (3, 28). Viral supernatants from clones 1C3 and 1B4 which exhibited titers $>10^6$ on NIH 3T3 cells and resulted in expression of CD4 on the transduced fibroblasts were used in the experiments described below. The culture supernatant was harvested, passed through a 0.45-mm-pore-size filter, and stored at −70°C until needed.

Autologous EBV-immortalized B-LCL were prepared as previously described (39). One milliliter of high-titer retroviral supernatant was added to 1 to 10 million actively dividing LCL in the presence of 4 μg of Polybrene at 37°C, 5% CO_2. After 48 h, the cells were washed by centrifugation, resuspended in growth medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 50 U of penicillin per ml, 50 mg of streptomycin per ml, and 2.0 mM glutamine), and selected by culture with 1.5 mg of G418 per ml. Cells expressing CD4 were further enriched from the drug-resistant LCL population by positive selection on CD4 MicroCELLector flasks (Applied Immune Sciences, Inc., Menlo Park, Calif.). CD4 expression by the transduced cells was evaluated by flow cytometry after labeling the LCL with fluorescein isothiocyanate (FITC)-conjugated anti-Leu-3A monoclonal antibody (Becton-Dickinson, San Jose, Calif.) or with the irrelevant FITC-conjugated MAb MOPC 21 (goat anti-mouse immunoglobulin G1 [IgG1], kindly provided by E. Clark, University of Washington, Seattle). The purified LCL transduced with CD4 were maintained in culture at 37°C in growth medium supplemented with 0.5 mg of G418 per ml. On average, more than 50% of the transduced cells remained $CD4^+$ after 3 months of culture. Positive selection on the anti-CD4-coated flasks was repeated at monthly intervals to prevent the potential outgrowth of a subpopulation of $CD4^-$ cells.

One million LCL-CD4 in 1 ml of growth medium were infected with 200 50% tissue culture infective doses of HIV-1$_{LAI}$, HIV-1$_{SF-2}$, or HIV-1$_{MN}$ (the latter two kindly provided by K. Steimer, Chiron Corporation) in 24-well plates (Costar, Cambridge, Mass.) and washed within 48 h. HIV-1 infection was analyzed by p24 antigen capture (Abbott Laboratories, Abbott Park, Ill.) of culture supernatant. To evaluate the percentage of infected cells, fluorescence-activated cell sorter (FACS) analysis for p55 core antigen expression in the cytoplasm of permeabilized cells was performed by utilizing murine MAb KC57-PE (Coulter Corp, Hialeah, Fla.) according to the product instructions. HIV-1 envelope expression was determined by FACS analysis of membrane staining with mouse MAb 110-4, an IgG1-recognizing HIV-1$_{LAI}$ gp120 (kindly provided by S.-L. Hu, Bristol-Myers Squibb Research Institute, Seattle, Wash.), and FITC-conjugated goat anti-mouse Ig (TAGO, Burlingame, Calif.). Cell-surface expression of the HIV-1 envelope was further verified by measuring under phase microscopy the ability of LCL-CD4 infected with HIV-1 (LCL-CD4$_{HIV-1}$ denotes infection with HIV-1$_{LAI}$ strain unless otherwise indicated) to form syncytia with SupT1 cells according to a modified procedure previously described (32). LCL-CD4$_{HIV-1}$ were incubated with SupT1 cells in a 1:10 ratio in microtiter plates, and syncytia were easily visualized by inverted phase microscopy within 6 h. No syncytia were visualized in wells containing uninfected LCL-CD4 and SupT1 cells. The production of infectious virions by LCL-CD4$_{HIV-1}$ was determined by adding filtered culture supernatants to CEM cells, washing the cells twice one h later, and testing the culture supernatants of the CEM cells for HIV-1 infection by p24 antigen production. Based on these infectivity studies, autologous LCL-CD4$_{HIV-1}$ were used as target or stimulator cells after 4 or more days of infection.

Effector cells. PBMC were isolated from heparinized blood by Ficoll-Paque density centrifugation as previously described (4), and effector cells for CTL assays were fresh PBMC either

tested in a direct assay or following in vitro stimulation to amplify HIV-1-specific $CD8^+$ effectors. PBMC were stimulated in vitro with one of three types of autologous stimulator cells: LCL-$CD4_{HIV-1}$, PBMC infected with a recombinant vaccinia virus (multiplicity of infection of 10) expressing either the HIV-1_{LAI} *env* (v-*env*) or HIV-1_{LAI} *gag* (v-*gag*) gene product (18, 19) (kindly provided by S.-L. Hu) or macrophages infected with the monocytotropic strain HIV-1_{Ba-L} (11). A total of 20×10^6 PBMC were stimulated with 2×10^6 LCL-$CD4_{HIV-1}$, $PBMC_{v-env}$, or $PBMC_{v-gag}$ for 9 days at 37°C, 5% CO_2 in a T25 flask, without supplemental exogenous recombinant interleukin 2. Alternatively, PBMC (2×10^6 per well) were stimulated for two 7-day intervals without supplemental recombinant interleukin 2 at 37°C, 5% CO_2 in 24-well plates with autologous adherent macrophages (2×10^5 per well), previously infected for 1 week with HIV-1_{Ba-L}, as previously described (5, 27).

Target cells. Target cells for assessing cytolytic activity included LCL-CD4, LCL-$CD4_{HIV-1}$ (infected with LAI, SF-2, or MN strains), and LCL or LCL-CD4 infected for 90 min with either v-*env*, v-*gag*, or vaccinia virus (NY strain), as previously described (5). No interference with expression of HIV-1 gene products has been detected in the CD4-transduced LCL infected with vaccinia virus recombinants. A total of 2×10^6 target cells were labeled overnight with 100 mCi of $^{51}NaCrO_4$ and were washed prior to use in the cytotoxicity assay.

Cytotoxicity assays. Standard 4-h chromium release assays were performed in triplicate by using mixtures of 10^4 target cells with varied numbers of effector cells in a total volume of 200 ml. The maximum spontaneous ^{51}Cr release in the absence of effector cells was less than 25% of maximal release by detergent lysis.

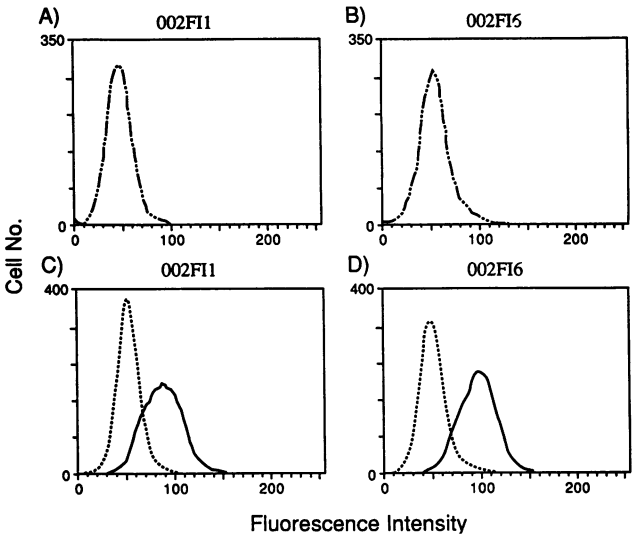

FIG. 1. Cell-surface expression of CD4 in LCL and LCL transduced with CD4 from two donors. Single-cell suspensions of 10^5 cells were stained with FITC-conjugated anti-Leu-3A (anti-CD4) or FITC-conjugated MOPC21 (goat anti-mouse IgG1) monoclonal antibodies, and fluorescence intensity was measured by flow cytometry. The gates were set to exclude probable nonviable cells and debris. The histograms represent fluorescence intensity on the *x* axis and cell number on the *y* axis. Staining with anti-Leu-3A is represented by the solid line (——), and staining with MOPC21 by the dashed line (– – –), and the lines in panels A and B are superimposable. (A) LCL from subject 002FI1. (B) LCL from subject 002FI6. (C) LCL-CD4 from subject 002FI1. (D) LCL-CD4 from subject 002FI6.

RESULTS

Characteristics of LCL transduced with the CD4 gene and infected with HIV-1. LCL from 13 donors were transduced with the human CD4 gene by culture with viral supernatants produced with the LT4SN retroviral vector and were positively and negatively selected as described in Materials and Methods. CD4 expression of transduced cells was evaluated by fluorescence after exposure to FITC-conjugated anti-Leu-3A. The CD4 gene was transduced successfully for all 13 donors tested, and the analysis of CD4 expression from cells from two representative donors is shown in Fig. 1. LCL from the two donors demonstrated no significant expression of CD4 prior to transduction (Fig. 1A and B). In contrast, more than 98% of the CD4-transduced LCL from both donors expressed CD4, as evidenced by a shift in the fluorescence histogram following staining with antibody to CD4 (Fig. 1C and D). Similar levels of CD4 expression were observed in the CD4-transduced LCL from the other 11 donors. Repeated analyses over time revealed that CD4 expression remained stable in G418-selected cells.

The susceptibility of the CD4-transduced LCL to HIV-1 infection was examined by measuring p24 antigen production in the culture supernatant of LCL-CD4 cells infected with 200 50% tissue culture infective doses of HIV-1. HIV-1 p24 antigen was detectable within 3 days of infection, with HIV-1_{LAI} in LCL-CD4 from three donors tested (Table 1). The production of p24 peaked within 2 weeks and remained elevated. Syncytium formation was not observed by inverted phase microscopy in cells containing infected LCL-CD4. HIV-1 infection in the CD4-transduced LCL from 8 of the 13 donors was examined, and the kinetics of p24 production was similar to that shown in Table 1 for the three donors. In comparison experiments, measurable levels of p24 antigen production detected in the nontransduced LCL were either absent or substantially delayed following in vitro infection.

The high levels of HIV-1 p24 antigen production in the culture supernatants following incubation of LCL-CD4 with HIV-1 confirmed that infection had occurred, but it was unclear if this resulted from a few cells producing large quantities of virus or from more uniform infection of the majority of cells. Although the presence of a small number of infected cells might permit the use of this population as stimulator cells for in vitro responses, the use of LCL-$CD4_{HIV-1}$ as targets for cytolytic assays requires that the majority of cells express the protein and be susceptible to lysis. Therefore, to determine the percentage of cells infected, LCL-$CD4_{HIV-1}$ were examined by flow cytometry for the presence of Gag proteins in the cytoplasm. Minimal nonspecific staining for Gag was detected in permeabilized HIV-1-uninfected LCL-CD4 (Fig. 2A and B). Within 4 days of

TABLE 1. Measurement of HIV-1_{LAI} infection by p24 antigen production in LCL transduced with CD4[a]

Volunteer	p24 antigen production (pg/ml) on postinfection day:			
	0	3	14	22
002FI1	<31	374	>600	>600
103AFIC	<31	92	>600	>600
103AFIB	<31	182	>600	>600

[a] One million LCL-CD4 were infected with 200 50% tissue culture infective doses of HIV-1_{LAI}, and culture supernatants were analyzed by p24 antigen capture. p24 antigen control values were <31 pg/ml, and 600 pg/ml was the maximum p24 antigen detectable by the assay.

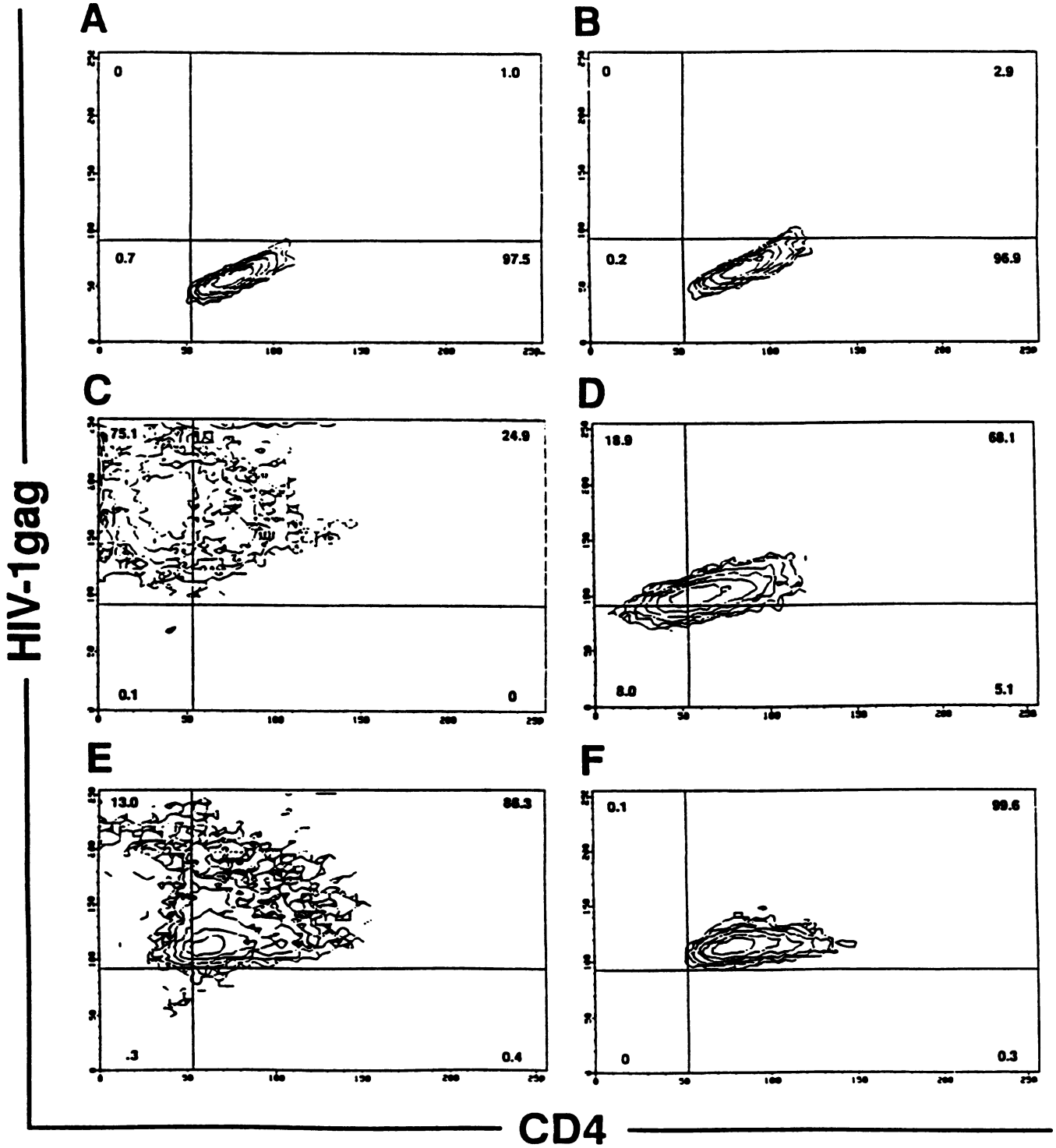

FIG. 2. Analysis by flow cytometry of HIV-1 infection and CD4 expression in CD4-transduced LCL (10^5 cells) from two donors, depicted by contour plots. LCL-CD4 before and after 4 days of infection with either HIV-1_{LAI} or HIV-1_{SF-2} were permeabilized, fixed, and stained with PE-conjugated KC57 (*y* axis), a murine monoclonal antibody to the HIV-1 core protein p55, and FITC-conjugated anti-Leu-3A (*x* axis). (A) Subject 002FI1, uninfected LCL-CD4. (B) Subject 002FI6, uninfected LCL-CD4. (C) Subject 002FI1, LCL-CD4 infected with HIV-1_{LAI}. (D) Subject 002FI6, LCL-CD4 infected with HIV-1_{LAI}. (E) Subject 002FI1, LCL-CD4 infected with HIV-1_{SF-2}. (F) Subject 002FI6, LCL-CD4 infected with HIV-1_{SF-2}.

infection with either HIV-1_{LAI} or HIV-1_{SF-2}, the majority of LCL-CD4 demonstrated Gag cytoplasmic staining (Fig. 2C to F). Only background or low levels of Gag cytoplasmic staining were detected in nontransduced LCL infected with HIV-1. Expression of envelope in LCL-$CD4_{HIV-1}$ was demonstrated by both immunofluorescence and the formation of syncytia with SupT1 cells, as described in Materials and Methods (data not shown). To investigate whether LCL-CD4 maintain their susceptibility to HIV-1 infection following cryopreservation, aliquots of LCL-CD4 were frozen in liquid nitrogen for more than 1 month, thawed, and then infected with HIV-1 as described above. The pattern of infection and HIV-1 gene expression was similar to that observed with fresh cells (data not shown). Thus, CD4 gene expression is stable in fresh and frozen LCL-CD4, and such cells are uniformly susceptible to infection with HIV-1.

The effect of HIV-1 infection on CD4 expression of the transduced LCL was determined, since $CD4^+$ cells producing gp120 have been reported to down regulate surface CD4 expression (7, 21). LCL-CD4 prior to and following infection were double-labeled with anti-CD4 and anti-Gag MAb and were examined by flow cytometry. CD4 was present in double-labeled, uninfected LCL-CD4 (Fig. 2A and B). However, following in vitro infection with HIV-1, particularly with the LAI strain, CD4 expression was reduced in a large portion of the transduced LCL (Fig. 2C to F).

The ability of LCL-CD4 to support productive infection was evaluated by assessing the supernatants of HIV-1-infected LCL-CD4 for the presence of infectious virus. Supernatants derived from cultured LCL-$CD4_{HIV-1}$ 4 days after infection were filtered and transferred to uninfected LCL-CD4 and CEM cells, and the production of p24 antigen was measured. On day 4 after infection with the culture supernatants, LCL-CD4 and CEM cells produced 332 and $\geq$600 pg/ml of p24, respectively, with control values of $\leq$60 pg/ml and maximum values of $\geq$600 pg/ml. Thus LCL-CD4 are susceptible to infection with vaccine prototype strains of HIV-1, express HIV-1 genes, and produce infectious virions.

Use of LCL-$CD4_{HIV-1}$ as target cells for CTL generated from HIV-1 vaccines. CTL recognizing envelope-expressing targets have been previously detected in an HIV-1-seronegative vaccine recipient, designated 002FI1, who had been immunized with a recombinant vaccinia virus expressing HIV-1 gp160 and had been boosted with recombinant gp160 (5, 27). To determine if HIV-1-infected LCL-CD4 could be recognized by such envelope-specific effectors, PBMC were obtained from the vaccinee and stimulated in vitro for two 1-week cycles with HIV-1-infected autologous macrophages. The cytolytic effector cells generated were tested either unfractionated or following enrichment for $CD8^+$ T cells for lysis of autologous LCL-$CD4_{HIV-1}$ targets. To determine that these target cells were lysable only by appropriate effector cells, PBMC from two seronegative individuals who had never received an HIV-1 vaccine and from another seronegative vaccine recipient who had not previously demonstrated any CTL activity to envelope-expressing targets were stimulated under similar conditions and tested for lytic activity. No CTL activity was detected from these control individuals against autologous LCL-CD4 infected with HIV-1_{LAI}. Results from a representative CTL assay for one of the unvaccinated individuals are shown in Fig. 3A. In contrast, effectors from vaccine recipient 002FI1, in whom we had previously detected envelope-specific CTL, demonstrated specific lysis of autologous HIV-1-infected LCL-CD4 targets at an effector-to-target ratio (E/T) as low as 5:1, with minimal lysis of the uninfected autologous LCL-CD4 target (Fig. 3B). $CD8^+$ rather than $CD4^+$ T cells were primarily responsible for the lytic activity (Fig. 3B), as demonstrated after enrichment using antibody-coated plates. Thus, LCL-$CD4_{HIV-1}$ appear useful as autologous target cells to determine if the CTL activity induced by candidate HIV-1 vaccines can lyse HIV-1-infected cells.

The analysis of CTL responses to individual HIV-1 proteins in vaccine recipients has been complicated by the presence of high levels of vaccinia-virus-specific lytic activity which interferes with the use of target cells expressing HIV-1 genes following infection with recombinant vaccinia viruses. This problem with identification of CTL responses has been exaggerated when recombinant vaccinia viruses have been used to elicit the in vivo and in vitro responses. Therefore, we examined whether the *env*-specific CTL precursors present in PBMC from the previously described vaccinee 002FI1 could be amplified in vitro by stimulation with v-*env*-infected autologous PBMC and detected by testing for lytic activity with HIV-1-infected LCL-CD4. To ensure that the recognition and lysis of CD4-LCL_{HIV-1} reflected specific activation of primed precursors and not an in vitro primary response, PBMC were similarly stimulated with v-*gag*-infected PBMC. Minimal lysis was detected against the LCL-$CD4_{HIV-1}$ targets by effectors that were generated following stimulation with v-*gag*-infected cells, suggesting that lytic activity reflected neither T-cell effectors generated by primary in vitro stimulation nor nonspecific effectors activated by vaccinia virus (Fig. 4A). However, v-*env*-stimulated effectors lysed LCL-$CD4_{HIV-1}$ targets at an E/T as low as 5:1, and did not lyse uninfected LCL-CD4 targets (Fig. 4A). The MHC restriction of these CTL responses was evaluated by testing the effector population against both autologous and allogeneic (mismatched at all class I and class II loci) LCL-$CD4_{HIV-1}$ and LCL-CD4 targets. Although some lytic activity was detected against the allogeneic LCL-$CD4_{HIV-1}$ at higher E/Ts (16% at an E/T of 40:1), presumably reflecting the presence of small numbers of nonspecific cytolytic effectors such as NK cells or ADCC activity, this was significantly lower than the lytic activity observed for the autologous LCL-$CD4_{HIV-1}$ target (37% at an E/T of 40:1) (Fig. 4B). These results indicate that use of LCL-$CD4_{HIV-1}$ may provide a means to more efficiently analyze HIV-1-specific CTL responses in recipients of recombinant vaccinia virus vaccines.

Use of LCL-$CD4_{HIV-1}$ as target cells to assess CTL activity in seropositive individuals. Our studies demonstrated that LCL-$CD4_{HIV-1}$ produce infectious virions and express multiple HIV-1 genes and thus should be useful as targets to assess the broad range of responses to many HIV-1 gene products expressed in infected cells in HIV-1-infected individuals. Therefore, PBMC from two HIV-1-seropositive individuals (103AFIC and 103AFIB) were stimulated with either v-*env*- or v-*gag*-infected autologous PBMC and were tested for lytic activity against autologous LCL-CD4 and LCL-$CD4_{HIV-1}$. Lysis of the uninfected LCL-CD4 was minimal at an E/T of 20:1, whereas CTL reactive to *env* and *gag* gene products expressed by the autologous LCL-$CD4_{HIV-1}$ target cells from both donors were demonstrable following amplification with v-*env*- and v-*gag*-infected stimulators, respectively (Fig. 5).

Direct assays of CTL responses in fresh PBMC have been useful for monitoring immunity in HIV-1-infected individuals. To evaluate whether LCL-$CD4_{HIV-1}$ could be used as targets in direct CTL assays in HIV-1 seropositive individuals, freshly isolated PBMC were tested for lytic activity against LCL-$CD4_{HIV-1}$ and against LCL-CD4 expressing HIV-1 genes in recombinant vaccinia viruses. A representative experiment from an HIV-1-infected individual (103AFIB) is shown in Fig. 6. No lytic activity was detected against the uninfected LCL-

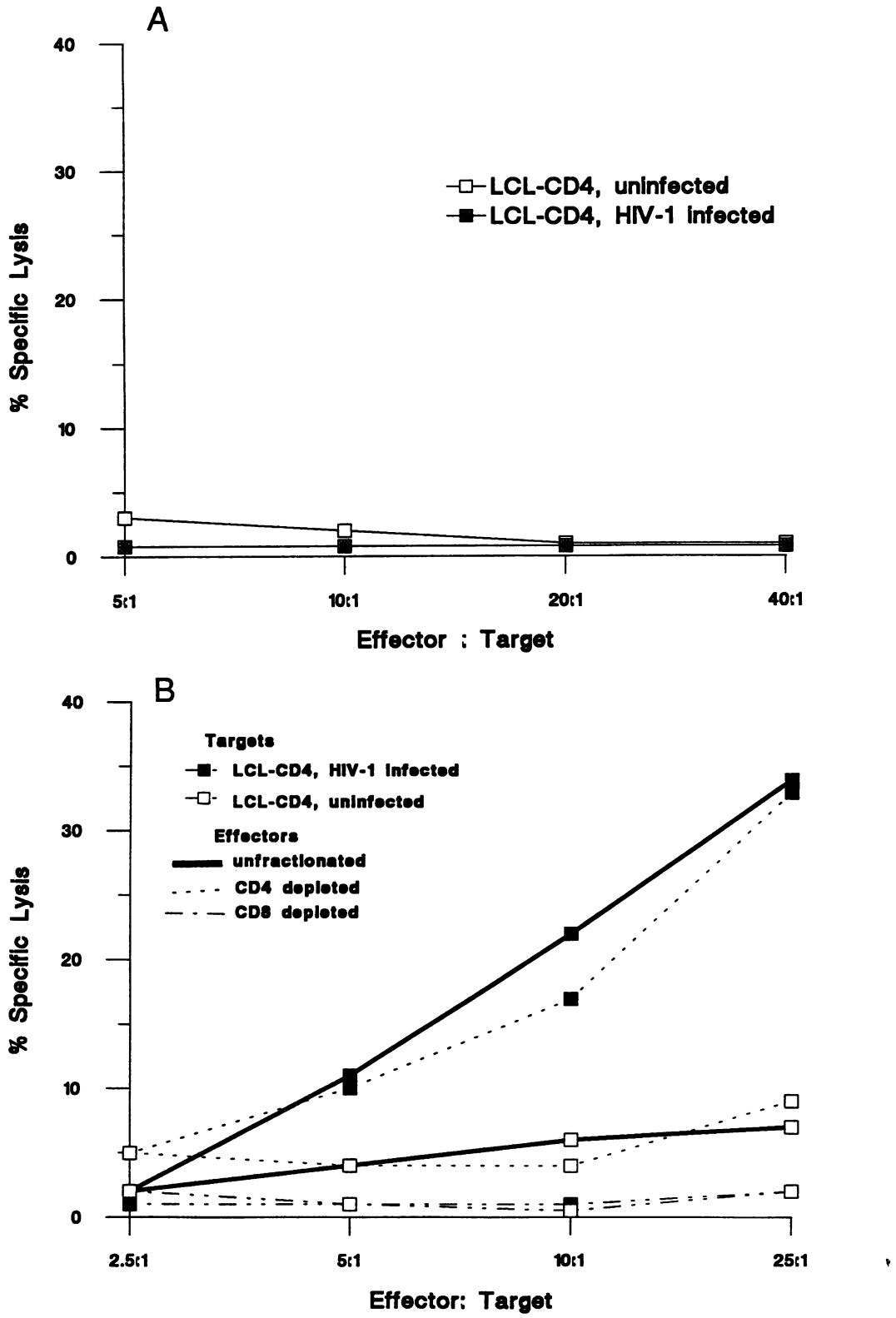

FIG. 3. Evaluation of HIV-1-specific CTL in two HIV-1-uninfected individuals. CTL precursors were stimulated for 2 weeks with HIV-1-infected macrophages and were tested at a wide E/T in a standard 4-h chromium release assay. (A) HIV-1-specific CTL in an individual who had never received an HIV-1 candidate vaccine. Effectors were tested against uninfected or HIV-1-infected (strain SF-2 or strain MN) LCL-CD4. (B) HIV-1-specific CTL from an individual (002FI1) receiving recombinant vaccinia virus containing HIV-1_{LAI} gp160 followed by rgp160 boosting. Effectors, including unfractionated, CD4-depleted, and CD8-depleted populations, were tested against autologous CD4-transduced LCL targets, either uninfected or HIV-1_{LAI}-infected.

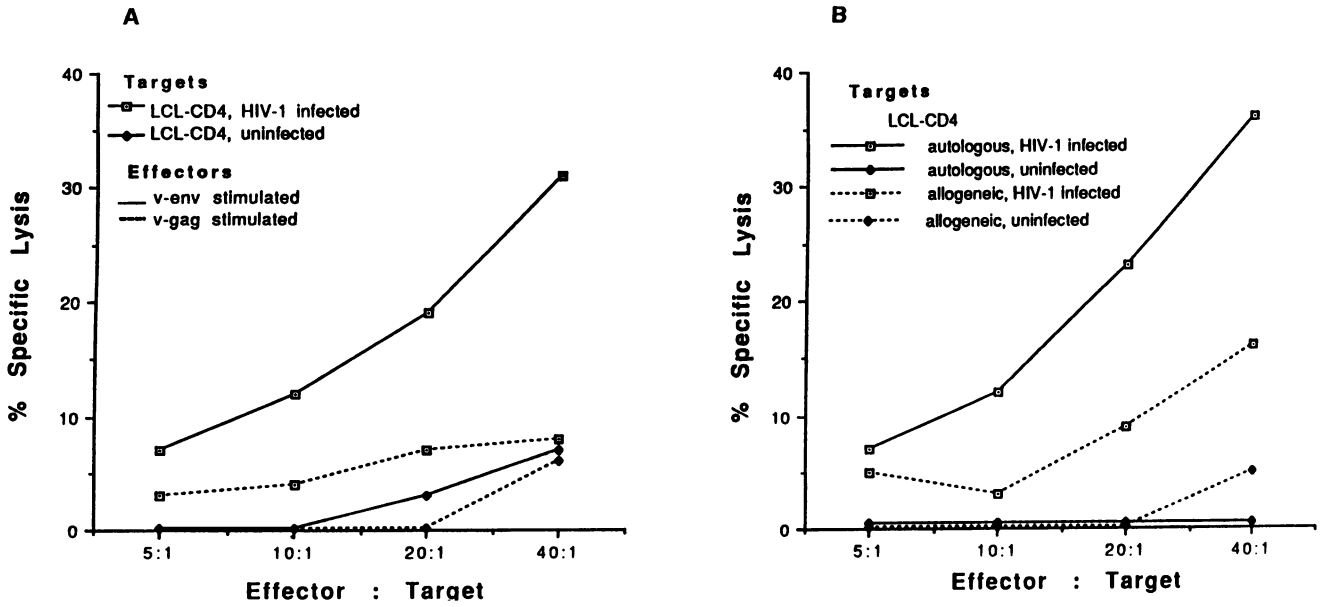

FIG. 4. Analysis of CTL against HIV-1-infected targets in a seronegative vaccinee (002FI1) by in vitro stimulation of effectors for 9 days with autologous PBMC infected with v-*env* or v-*gag*. (A) Autologous LCL-CD4 targets were either uninfected or infected with HIV-1_{LAI}. (B) MHC-restricted CTL activity was assessed by the ability of the v-*env*- or v-*gag*-stimulated effectors to lyse autologous versus allogeneic LCL-CD4 targets (mismatched at all class I and II loci) that were either uninfected or infected with HIV-1_{LAI}.

CD4, affirming that expression of HIV-1 genes in the target cell was essential for detection of lytic activity. Specific lysis was demonstrated in this direct assay against the HIV-1-infected LCL-CD4 at an E/T as low as 25:1. Moreover, no direct lytic activity against v-*env*- or v-*gag*-infected LCL was detected in this assay, despite the fact that such targets are efficiently recognized by in vitro activated CTL (27). Similar results in which LCL-$CD4_{HIV-1}$ were more sensitive to lysis by unstimulated effectors obtained directly from peripheral blood lymphocytes in two other HIV-1-infected individuals were observed. These data suggest that HIV-1-infected LCL-CD4 may be useful as targets cells in direct assays of activated cytolytic effectors from the peripheral blood of seropositive individuals and may improve sensitivity, since lysis of this target reflects the collective activities of effectors recognizing all HIV-1 gene products.

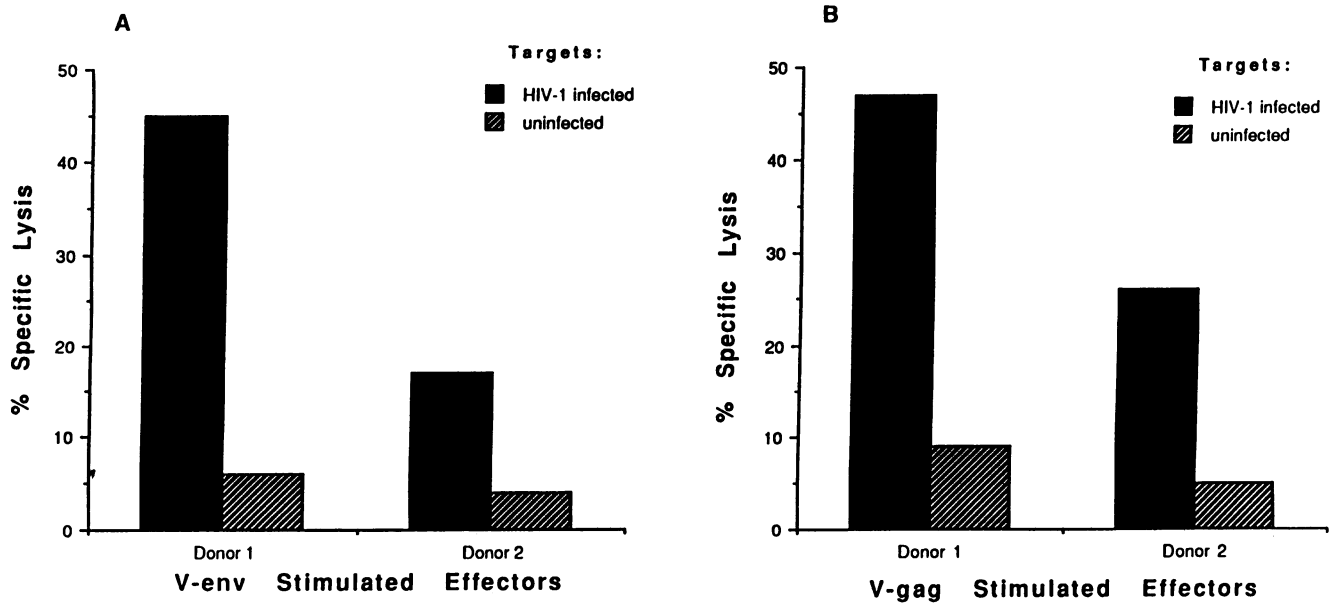

FIG. 5. CTL responses against LCL-CD4 targets, uninfected or infected with HIV-1_{LAI}, in two HIV-1-infected subjects: donor 1, 103AFIC, and donor 2, 103AFIB. Bulk PBMC were stimulated with autologous PBMC infected with either v-*env* or v-*gag* for 9 days. Percent specific lysis is presented at an E/T ratio of 20:1 and shown for v-*env*-stimulated effectors (A) and v-*gag*-stimulated effectors (B).

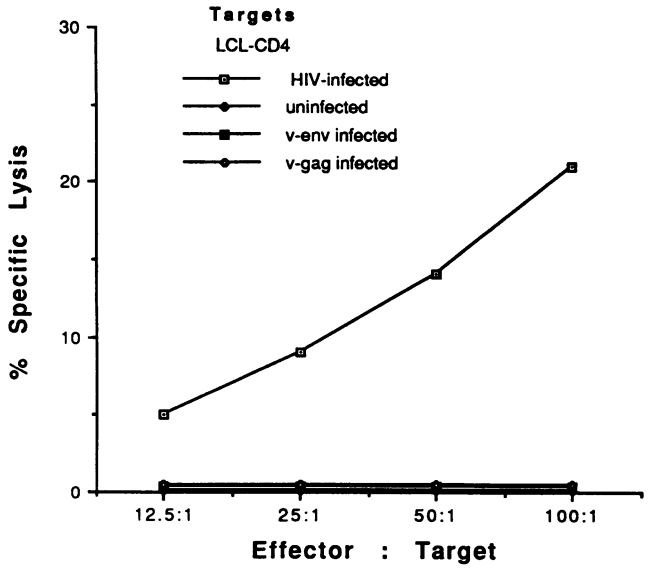

FIG. 6. Direct CTL analysis in an HIV-1 infected individual, 103AFIB. Fresh PBMC were tested at multiple E/Ts in a standard 4-h chromium release assay against autologous LCL-CD4 uninfected or infected with v-*env*, v-*gag*, or HIV-1_{LAI}.

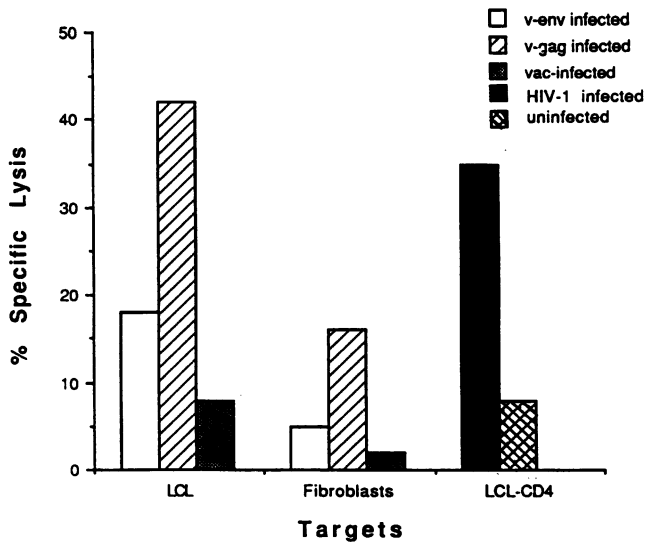

FIG. 7. Use of LCL-CD4$_{HIV-1}$ as stimulator cells to amplify HIV-specific CD8$^+$ CTL precursors from an HIV-1-infected subject, 103AFIC. The effector population was stimulated with autologous LCL-CD4$_{HIV-1}$ for 9 days and was enriched for CD8$^+$ cells by adherence to anti-CD4-coated flasks prior to testing. Autologous targets tested include LCL and fibroblasts infected with v-*env*, v-*gag*, and *vac* and LCL-CD4, either uninfected or infected with HIV-1_{LAI}. Percent specific lysis is presented at an E/T of 20:1.

LCL-CD4$_{HIV-1}$ as stimulator cells to induce CTL responses. The ability of LCL-CD4$_{HIV-1}$ to function as stimulator cells for the amplification of HIV-specific CTL precursors was also examined. PBMC obtained from both seropositive and seronegative vaccinees were stimulated with LCL-CD4$_{HIV-1}$ and tested for lysis against LCL and fibroblasts infected with either v-*env*, v-*gag*, or *vac*, and uninfected or HIV-1-infected LCL-CD4. PBMC from the seropositive individual 103AFIC, stimulated with LCL-CD4$_{HIV-1}$ and enriched for CD8$^+$ effector cells, lysed autologous LCL and fibroblasts expressing HIV-1 *gag* (Fig. 7). (Lysis of target cells expressing HIV-1 gene products was not detected in a direct assay of fresh unstimulated PBMC.) Substantially lower levels of lytic activity were observed with target cells expressing HIV-1 *env* rather than HIV-1 *gag*, consistent with several studies suggesting that *gag* is the major target of the CD8$^+$ CTL response in seropositive individuals (12). The vaccinia-virus-infected LCL were more readily lysed as targets than were fibroblasts. In addition, HIV-1-infected LCL-CD4, but not the uninfected LCL-CD4, were lysed by the induced effector population, suggesting that an HIV-1-specific response could be distinguished from a response to EBV expressed in the LCL.

In studies with PBMC derived from seronegative vaccinees, the LCL-CD4$_{HIV-1}$ proved to be unsuitable as stimulator cells because of the presence of strong EBV-specific responses. Induction of high levels of EBV-reactive lytic activity was detected in these cultures, resulting in high background lysis against the *vac*-infected LCL and uninfected LCL-CD4 targets (data not shown). Thus, the use of LCL-CD4$_{HIV-1}$ as stimulator cells may be restricted to assays designed to amplify effector cells from individuals who lack sufficient immunocompetence to generate strong EBV-specific responses.

DISCUSSION

This report describes a method to produce human autologous cell lines that can be reproducibly infected with HIV-1. By transducing EBV-transformed LCL with the LT4SN retroviral vector, it was possible to generate stable B-cell lines expressing CD4, and such LCL-CD4 became susceptible to infection with several HIV-1 strains. Following infection, the LCL-CD4$_{HIV-1}$ remained viable for more than 2 weeks, expressed multiple HIV-1 genes, and produced infectious virions. The ability to produce such autologous infectable permanent cell lines provides many opportunities for the study of HIV-1-specific immunity.

The analysis of CTL responses in uninfected, immunocompetent HIV-1 vaccine recipients participating in several clinical trials (5, 15) has been obstructed by the paucity of shuttle vectors available to efficiently express HIV-1 genes in autologous cells. The most common approach for detecting HIV-1 envelope-specific CTL in the recipients of envelope vaccines has been to use autologous cells infected with vaccinia virus recombinants expressing HIV-1 envelope gene products as stimulator and target cells. Frequently, the envelope-specific CTL responses have been difficult to distinguish from the high-level vaccinia-virus-specific CTL responses in the immunocompetent individuals with previous vaccinia virus exposure or who have been recently immunized with recombinant vaccinia virus vaccines. The LCL-CD4$_{HIV-1}$ described in this study express no vaccinia virus antigens and thus can potentially circumvent this problem. Our results demonstrate that, following amplification of PBMC with v-*env*-infected stimulators, even in individuals recently immunized with a recombinant vaccinia virus, LCL-CD4$_{HIV-1}$ proved to be sensitive targets to detect MHC-restricted HIV-1-specific CD8$^+$ CTL without interference by vaccinia virus responses.

LCL-CD4 may have increased utility with the next generation of candidate HIV-1 vaccines, which will likely contain multiple HIV-1 gene products in the context of either a recombinant virus, a particle containing several HIV-1 proteins, or possibly an attenuated virus (8, 16). Thus, it may be necessary to test recipients for CTL responses to multiple

HIV-1 gene products. Our results demonstrate that LCL-$CD4_{HIV-1}$ can be employed as a single target to detect CTL effector responses in HIV-1-seropositive individuals to several HIV-1 gene products. Therefore, for analysis of responses to complex vaccines, HIV-1-infected LCL-CD4 may prove useful as a screening target, and, if lytic activity is demonstrated, subsequent detailed analyses of activity against specific HIV-1 proteins could be performed.

LCL-CD4 should be helpful for monitoring CTL activity in HIV-1-infected individuals. A decline in CTL responses to HIV-1-specific proteins in seropositive individuals as HIV-1 disease progresses (14, 17) has been demonstrated, but little information is available concerning the extent to which lytic activity is lost against HIV-1-infected cells. Although these are presumably related, the analysis of the response to a single protein will vary in individuals possessing different MHC haplotypes and infected with different strains. Thus, longitudinal monitoring of changes in CTL activity against HIV-1-infected cells such as LCL-$CD4_{HIV-1}$ may provide a more accurate means of assessing the relationship of the decline in CTL responses and disease progression. In addition to using LCL-$CD4_{HIV-1}$ in such studies of the natural history of HIV-1 infection, it may be informative to include it in the panel of target cells used to evaluate augmentation of immune responses following immunotherapy of seropositive individuals, particularly if, as in previous studies, the immunogen is a recombinant protein expected to predominantly augment $CD4^+$ helper responses. In this setting, the augmentation of the helper response may be expected to amplify all CTL responses, and thus it will be necessary to evaluate the full spectrum of $CD8^+$ T cells specific for HIV-1 proteins (23).

It has been well documented that many HIV-1 mutations result from the frequent errors in reverse transcription, and that some of these mutants may subsequently be selected in treated hosts because of resistance to antiretroviral therapy (24, 36) or through loss of the epitope recognized by autologous neutralizing antibodies (1, 2). Similarly, viral mutants may evade elimination by CTL in HIV-1-infected individuals through an alteration in the epitopes that interact with the presenting class I MHC molecule or the T-cell receptor recognizing the MHC-peptide complex (20, 35). However, the importance of viral escape from recognition by host CTL in disease progression has been difficult to assess, since it has required molecular mapping of the epitope from an initial viral isolate recognized by host CTL, followed by isolation of virus during periods of disease progression for cloning and sequencing of the relevant gene (35). Longitudinal monitoring of CTL responses in HIV-1-infected individuals might be more simply achieved by studying CTL reactivity with autologous LCL-CD4 infected with serial autologous HIV-1 isolates. In this situation, the failure of a viral isolate to sensitize a target for lysis by the CTL would imply that epitope selection had occurred as one possibility, and in only selected instances would this need to be confirmed by cloning and sequencing. In this paper we have demonstrated that LCL-CD4 can be infected with several laboratory strains of HIV-1, and future studies will need to be performed to determine the efficiency with which clinical isolates can infect these target cells.

LCL-$CD4_{HIV-1}$ may also have certain advantages as stimulator cells in the amplification of HIV-1-specific CTL in HIV-1-infected individuals. CTL precursor responses to multiple HIV-1 gene products can be amplified with the use of a single stimulator cell, and it should thus be possible to analyze and quantify HIV-1-specific responses by using LCL-$CD4_{HIV-1}$ as stimulator cells in limiting dilution precursor frequency assays and for cloning. After such clones are generated, the fine specificity for individual HIV-1 proteins can be identified and confirmed by testing with targets expressing individual HIV-1 genes.

In conclusion, our studies describe a method to establish cell lines from any individual that can serve as an autologous source of highly susceptible HIV-1-infected cells. We believe that such LCL-$CD4_{HIV-1}$ will have broad utility in the evaluation of CTL responses induced by candidate vaccines designed to prevent HIV-1 infection and in the study of the natural history of HIV-1 infection and immunological intervention with seropositive individuals.

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