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Direct Injection of a Recombinant Retroviral Vector Induces Human Immunodeficiency Virus-Specific Immune Responses in Mice and Nonhuman Primates

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The cytotoxic T-lymphocyte (CTL) response plays an important role in controlling the severity and duration of viral infections. Immunization by direct in vivo administration of retroviral vector particles represents an efficient means of introducing and expressing genes and, subsequently, the proteins they encode in vivo in mammalian cells. In this manner foreign proteins can be provided to the endogenous, class I major histocompatibility complex antigen presentation pathway leading to CTL activation. A nonreplicating recombinant retroviral vector, encoding the human immunodeficiency virus type 1 (HIV-1) IIIB envelope and rev proteins, has been developed and examined for stimulation of immune responses in mouse, rhesus macaque, and baboon models. Animals were immunized by direct intramuscular injection of the retroviral vector particles. Vector-immunized mice, macaques, and baboons generated long-lived CD8$^+$, major histocompatibility complex-restricted CTL responses that were HIV-1 protein specific. The CTL responses were found to be dependent on the ability of the retroviral vector to transduce cells. The vector also elicited HIV-1 envelope-specific antibody responses in mice and baboons. These studies demonstrate the ability of a retroviral vector encoding HIV-1 proteins to stimulate cellular and humoral immune responses and suggest that retrovector immunization may provide an effective means of inducing or augmenting CTL responses in HIV-1-infected individuals.

In many viral infections the cellular immune response plays an important role in eliminating virus-infected cells. Cytotoxic T lymphocytes (CTL) specifically lyse infected cells and are important in limiting the severity and duration of many viral diseases (11–13, 18, 28, 29, 31). Many individuals infected with the human immunodeficiency virus type 1 (HIV-1) exhibit cellular immune responses. CTL specific for different HIV-1 proteins have been found in HIV-1 seropositive individuals (7, 14, 23, 33, 38–40). Specific CTL have also been demonstrated early in the course of HIV infection, prior to the appearance of antibody, and are presumed to control the initial viremia often observed early in the disease (37). The presence of specific CTL in seropositive individuals has been associated with slower progression to AIDS (5, 16). Because of the potential role of CTL in controlling HIV-1 infection and disease, numerous approaches have been suggested for activating the cellular immune response including inactivated virus (9), subunit vaccines (1, 2), and viral vectors (42).

Many conventional immunization procedures involving the administration of recombinant proteins or killed virus preparations are currently being evaluated to combat HIV infection and disease. These procedures primarily deliver foreign proteins to the exogenous class II major histocompatibility complex (MHC) antigen presentation pathway. The class II pathway favors activation of CD4$^+$ T cells leading to antibody production (8). Although soluble proteins have been shown to induce CTL activity (10, 15), efficient activation of class I-restricted, CD8$^+$ CTL generally occurs by intracellular synthesis of foreign proteins and subsequent antigen processing and presentation through the endogenous, class I presentation pathway (8, 20). Retrovector immunization is a means of introducing foreign genes into the genome of host cells and, subsequently, foreign proteins into the intracellular antigen-processing compartment. This should provide these immunogens for class I MHC-restricted antigen presentation (41), favoring stimulation of CD8$^+$ CTL.

We have been studying the induction of CTL responses specific for HIV-1 proteins in mice and nonhuman primates using immunization with a recombinant retroviral vector. This nonreplicating vector (N2 IIIBenv) encodes the envelope (env) and rev proteins from HIV-1 IIIB and has previously been employed to provide endogenous production of these HIV-1 proteins for antigen presentation in murine and nonhuman primate cells. These studies have demonstrated the induction of antigen-specific, class I MHC-restricted, CD8$^+$ CTL responses in mice and rhesus monkeys following injection of ex-vivo vector-transduced autologous fibroblasts (6, 17, 41). Furthermore, HIV-infected patients treated with vector-transduced autologous fibroblasts have shown augmented HIV-1 IIIB env-specific CD8$^+$ CTL responses (43).

As an extension of these studies, we have examined vector immunization involving direct administration of the N2 IIIB-env retroviral vector. Direct, in vivo administration of the vector provides a more feasible approach for retrovector immunization compared with using ex-vivo-transduced cells. This study demonstrates that HIV-1-specific CD8$^+$ CTL responses are induced in mice, rhesus monkeys, and baboons following administration of this retroviral vector. In addition, antibody responses are elicited in mice and baboons following vector immunization. This study represents the first direct

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administration of a recombinant retroviral vector leading to the induction of a cell-mediated immune response.

MATERIALS AND METHODS

Animals. Six- to eight-week old, female BALB/c mice (*H-2*^{d}), obtained from Harlan Sprague-Dawley (Indianapolis, Ind.), were used in these experiments. Rhesus macaques and baboons were maintained at White Sands Research Center (Alamogordo, N. Mex.). Blood samples from the rhesus macaques and baboons were obtained at White Sands and shipped on ice via overnight air delivery for testing.

Retroviral vectors. The nonreplicating, amphotropic murine N2 IIIBenv retroviral vector carries the HIV-1 IIIB env and rev genes and a neomycin phosphoryltransferase gene (Neo^r) conferring resistance to the antibiotic geneticin (G418) (6). The HIV-1 env and rev genes were excised from this provector by digestion with *Xho*I and *Cla*I, and this vector backbone, which retained the Neo^r marker, was used in the construction of additional retroviral vectors described below. The N2 III Brev vector, which encodes HIV-1 rev and Neo^r, was generated by inserting the rev cDNA coding sequence from pcREV (19) (a generous gift from B. Cullen) into the N2 backbone at the *Xho*I-*Cla*I restriction sites. The bacterial *lacZ* gene encoding β-galactosidase (a generous gift from J. Kwang) (27) was cloned into the N2 backbone as described above to generate an N2 β-galactosidase vector.

Aliquots of vector (0.2 ml each) were tested for the presence of replication-competent retrovirus by an extended S^+/L^- assay (26). The vector-producing cell line and vector preparations were also tested by cocultivation with *Mus dunni* cells, and the supernatant generated was assayed for replication-competent retrovirus by a hygromycin marker rescue assay (22). All vector-producing cells and vector preparations were shown to be free of detectable replication-competent retrovirus by using these assay systems.

Vector production. Novel retroviral packaging cell lines have been developed for these studies on the basis of the expression of murine leukemia virus gag/pol and env sequences in the D-17 canine cell line (ATCC CCL 183). The packaging cell line (DA) was developed by the principle of splitting the retroviral genome, removing the viral long terminal repeat sequences, and replacing them with the cytomegalovirus immediate-early promoter (3) to reduce the potential of generating replication-competent retrovirus (37a). The DA/N2 IIIBenv producer cell line was generated by transduction of vesicular stomatitis virus G pseudotyped vector (4) into the DA packaging cell line. DA/N2 IIIBenv producer cells were grown, and the supernatant containing the replication-defective N2 IIIBenv vector was collected, filtered (pore size, 0.45 μm), and stored at −80°C. The vector titer was determined by serial dilution and CFU assay on HT 1080 indicator cells selected in G418-containing medium. Titers were typically between 10^6 and 10^7 CFU/ml.

CTL target cell development. The murine fibroblast cell line BC10ME (*H-2*^{d}) (25) was transduced with the previously described amphotropic recombinant retroviral vectors encoding HIV-1 IIIB env/rev, HIV-1 IIIB rev, or β-galactosidase, resulting in the development of the corresponding target cell lines BC-env/rev, BC-rev, and BC-βgal. The murine fibroblast cell line BL/6 (*H-2*^{b}) (34) was transduced with a recombinant retroviral vector encoding HIV-1 IIIB env/rev to generate the control target cell line BL/6-env/rev.

In certain experiments the murine effector cells were tested for lysis of BC target cells that were coated with peptide during radioisotope labeling. Either the p18 IIIB peptide (35) derived from the envelope gp120 V3 loop region of the HIV-1 IIIB virus isolate [amino acids 315 to 329; RIQRGPGRAFVT IGK(C); Chiron Mimotopes, Clayton, Australia] or the analogous peptide derived from the HIV-1 MN strain (36), p18 MN [amino acids 315 to 329; RIHIGPGRAFYTTKN(C)] was added (10 μM) to cells during radioisotope labeling. Following a 1-h incubation, peptide-coated target cells were washed four times to remove both unbound peptide and excess radioisotope. In other experiments, target cells infected with recombinant vaccinia virus vectors (Vac-gp160 and Vac-LacZ [generous gifts from B. Moss and D. Kuritzkes, respectively]) were employed as previously described (6).

Rhesus macaque and baboon peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation (Histopaque; Sigma, St. Louis, Mo.). Washed PBMC (4×10^6 per well) were added to 24-well plates (Corning, Corning, N.Y.) and transformed by the addition of purified herpesvirus papio derived from the 549S-1X1055 cell line (32) (a generous gift from Krishna Murthy) at a multiplicity of infection of 3:1 plus Polybrene (2 μg/ml) (Sigma). The established B lymphoblastoid cell lines (BLCL) were grown in RPMI 1640 medium supplemented with 20% fetal bovine serum (HyClone, Logan, Utah), 1 mM sodium pyruvate (Irvine Scientific, Santa Ana, Calif.), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Irvine Scientific), and 50 μg of gentamicin per ml (RPMI 20% growth medium). The nonhuman primate BLCL were vector transduced by cocultivation with a xenotropic producer cell line HX/N2 IIIBenv as a source of the N2 IIIBenv vector. The HX/N2 IIIBenv producer cells (10^6) were gamma irradiated (10,000 rads of ^{60}C) and added (in 2 ml of RPMI 20% growth medium) to 6-cm-diameter tissue culture dishes containing 10^6 BLC. The cultures were incubated for 5 days at 37°C in 5% CO_2, washed, and placed in selective medium (G418; 800 μg/ml). The transduced BLC were tested for HIV-1 env expression by Western blot (immunoblot) analysis.

Vector immunization. Mice were injected either intramuscularly (i.m.) with 0.2 ml (0.1 ml at each of two muscle sites) of the N2 IIIBenv vector (>10^6 CFU/ml) or intraperitoneally with 1 ml of vector, on day 0 and day 7. Seven days after the final injection mice were sacrificed and the spleens were removed. In vitro cultures of primed splenocytes were established as previously described (41). Briefly, splenocytes (3×10^6 cells per ml) were cultured for 7 days in vitro with gamma-irradiated (10,000 rads of ^{60}Co) BC-env/rev stimulator cells (6×10^4/ml) in 10 ml of RPMI growth medium containing 10% fetal bovine serum in T-25 flasks (Corning). The resulting effector cells were subsequently tested for cytotoxic activity. In certain experiments, murine CTL lines were generated and propagated by multiple in vitro restimulations. CTL effector lines (10^6 cells per ml) were restimulated with gamma-irradiated BC-env/rev cells (6×10^4/ml) and gamma-irradiated (3,000 rads) BALB/c splenocyte filler cells (2×10^6/ml) every 9 to 12 days. RPMI 10% growth medium (10 ml) was supplemented with recombinant human interleukin 2 (2 U/ml) Boehringer GmbH, Mannheim, Germany). CTL effector lines were assayed for cytotoxic activity 7 days after restimulation.

Rhesus monkeys and baboons were injected i.m. with up to a total of 3 ml of vector (10^7 CFU/ml) distributed to four to six different muscle sites. These animals were injected three times at 2-week intervals. Peripheral blood was collected prior to each injection, and PBMC were isolated from a Ficoll gradient. The PBMC (10^6/ml) were stimulated in vitro with irradiated (10,000 rads), autologous, vector-transduced BLC (10^6/ml) in T-25 culture flasks in RPMI 20% growth medium (10 ml). The resulting effector cells were harvested 7 days later and tested in a ^{51}chromium release cytotoxicity assay.

Cytotoxicity assay. The ^{51}chromium release cytotoxicity assay was performed for murine and nonhuman primate effector cells as described previously (17, 41). Briefly, the $Na_2{}^{51}CrO_4$-labeled syngeneic (murine) and autologous (macaque and baboon) target cells (10^4) were incubated for 4 h at 37°C with various numbers of effector cells (10^4 to 10^6) in a final volume of 200 μl in a 96-well round-bottom plate. Culture supernatants (100 μl) were harvested and assessed for specific ^{51}Cr release (counts per minute) by using a gamma spectrometer. Spontaneous release was determined as counts per minute from targets plus medium and was typically 10 to 20% of the maximum release. The maximum release was determined as counts per minute from targets plus 1 M HCl. Target cell lysis (percent) was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. In some cases, results are presented as percent net target cell lysis (specific target cell lysis − control target cell lysis).

T-cell depletion. Mouse cytotoxic effector cells were treated with CD4 or CD8 T-cell-specific monoclonal antibody and then with complement (Low-Tox; Cedarlane, Westbury, N.Y.) to deplete the respective T-cell populations, as previously described (41). Macaque cytotoxic effector cells were treated with CD8-specific monoclonal antibody (T8; Dako, Carpenteria, Calif.), washed, and treated with a fluoresceinated anti-mouse immunoglobulin (Cappel, Durham, N.C.). These CD8-enriched ($CD8^+$) and CD8-depleted ($CD8^-$) effector cell populations were isolated by flow cytometry. Baboon cytotoxic effector cells were incubated with a CD8-specific monoclonal antibody (Dynal, Great Neck, N.Y.). The cells were treated with anti-mouse immunoglobulin-conjugated magnetic beads (Dynal). Cells that bound the iron conjugate were separated with a magnet, yielding CD8-enriched and CD8-depleted effector cell populations. Effector cells were washed and then incubated with target cells.

Antibody detection. BALB/c mice were injected i.m. with 0.1 ml of the N2 IIIBenv vector and boosted twice at weekly intervals. Sera were collected (retro-orbital venous plexus during metaphane-induced anesthesia) prior to each injection. Baboons were injected with vector as described above, and sera were collected pre- and postimmunization. By using an enzyme-linked immunosorbent assay (ELISA), sera were examined for binding to purified HIV-1 IIIB gp120 protein (25 ng per well) (ABT, Cambridge, Mass.), and the assay was developed with the ABTS system (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and analyzed at 450 nm on a microplate reader (Molecular Devices, Menlo Park, Calif.).

RESULTS

The generation of HIV-1-specific CTL responses directed against the gene products delivered by the N2 IIIBenv vector (HIV-1 env, HIV-1 rev, and Neo^r) was evaluated in BALB/c mice. Splenocytes obtained from vector-immunized mice (2×10^5 CFU) were tested for cytolytic activity on syngeneic target cells expressing HIV-1 env and rev proteins and the Neo^r marker (BC-env/rev). HIV-1-specific cytotoxic activity from a representative individual mouse was demonstrated on BC-env/rev target cells but not control target cells (Fig. 1A). To indicate the approximate frequency with which N2 IIIBenv-induced CTL responses occur, Fig. 1D shows a compilation of recent experiments involving 76 vector-injected mice. This figure indicates the net specific target cell lysis at an effector/target ratio of 100:1 for each individual mouse tested and reveals that 64 of these mice (response frequency, 84%) generated net specific target cell lysis of greater than or equal to 15%.

The ontogeny of the N2 IIIBenv-induced CTL response in mice has a relatively rapid onset and is long-lived. Mice injected a single time and sacrificed 7 days later have occasionally exhibited specific CTL activity after a 7-day in vitro culture (data not shown); however, two vector administrations 1 week apart, followed by in vitro culture, generate CTL responses with a frequency of about 84% (Fig. 1D). The CTL response is relatively long-lived, as mice sacrificed 17, 20, 25, and 27 weeks after the final vector injection revealed net CTL responses of 50 to 99% at an effector-to-target ratio of 100:1 (data not shown). Two mice, sacrificed 36 weeks after the final vector administration, demonstrated relatively low net lysis (13 and 14% at 100:1), but the CTL responses in individual mice were not followed over time, so no conclusions can be made as to whether these mice possessed low CTL activity at all times postinjection or the CTL response was dropping to low levels at this extended time point.

Induction of cytotoxic activity by direct injection of the N2 IIIBenv retroviral vector was also examined in naive, healthy nonhuman primates. Rhesus macaques and baboons were injected with the N2 IIIBenv vector, and cellular immune responses specific for HIV-1 env/rev-expressing target cells were assessed. Both rhesus macaques and baboons (Fig. 1B and C, respectively) generated HIV-1 env/rev-specific cytotoxic responses after vector injection, as shown by the lysis of autologous HIV-1 env/rev-expressing target cells compared with nontransduced control targets in these selected individuals. Table 1 shows CTL responses from all vector-injected macaques and baboons at a single effector/target ratio and sampled at various time points during the study. PBMC obtained prior to vector injection from all treated macaques and baboons (week 0) and in vitro stimulated with vector-transduced cells did not lyse HIV env/rev-expressing autologous target cells, indicating that in vivo priming was necessary for induction of cytotoxic responses in these animals. These data indicate that six of six macaques and four of four baboons generated specific CTL responses (Table 1).

The ontogeny of the CTL response in nonhuman primates was assessed both directly and indirectly. Eight of the 10 nonhuman primates injected with N2 IIIBenv exhibited specific target cell lysis with the first postinjection blood sampling, 2 to 4 weeks following vector administration (Table 1). To directly examine the onset of specific cytotoxicity and the subsequent loss of this response, macaque R188 was injected once at four i.m. sites and peripheral blood lymphocytes were subsequently sampled for specific CTL activity. This animal showed no specific target cell lysis prior to vector administration but exhibited CTL activity 3 weeks after injection (Table 1). Macaque R188 maintained demonstrable specific target cell lysis 28 and 32 weeks postinjection, but the CTL response dropped by week 36.

The effector cell populations induced by N2 IIIBenv administration to mice and nonhuman primates were characterized for the CD4/CD8 T-cell phenotype. Results indicated that mouse, rhesus macaque, and baboon effector cell populations (Fig. 2A, B, and C, respectively) were $CD8^+$ $CD4^-$ CTL, as shown by abrogation of cytotoxic activity following depletion of $CD8^+$ T cells, whereas $CD4^+$ T-cell depletion (or enrichment of $CD8^+$ T cells) of the effector cell population had a negligible effect on the lysis of target cells compared with the lysis by untreated control effector cells.

The vector-induced $CD8^+$ CTL were subsequently evaluated for MHC restriction requirements. The HIV-specific BALB/c ($H\text{-}2^d$) mouse CTL recognized and lysed syngeneic

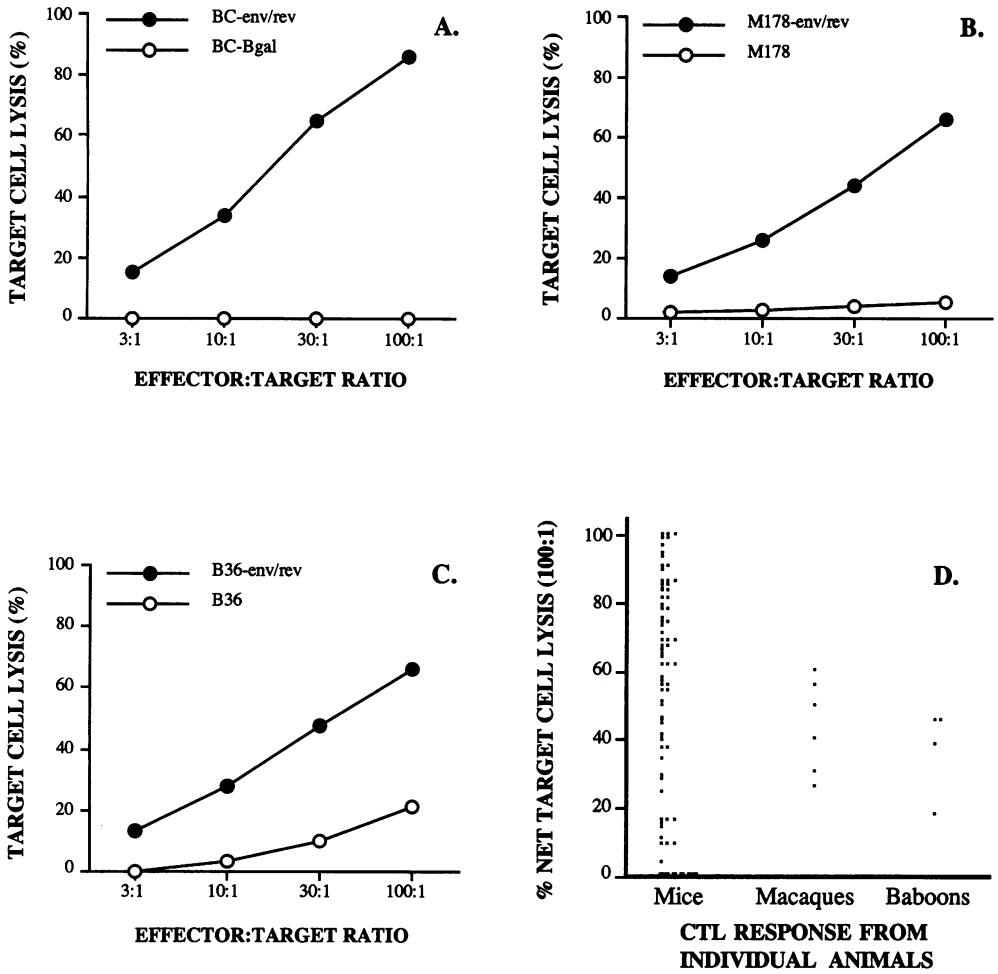


FIG. 1. Retrovector immunization with N2 IIIBenv induces cytolytic activity in mice and nonhuman primates. N2 IIIBenv vector-generated effector cells from a mouse (A), a macaque (B), or a baboon (C) were tested for lytic activity on target cells expressing HIV-1 env/rev antigens or control target cells. Syngeneic target cells were employed to test mouse effector cells (A), and autologous B lymphoblast targets were used to test macaque M178 (B) and baboon B36 (C) effector cells. The mean of the percent target cell lysis from triplicate assay wells is plotted at each effector-to-target cell ratio shown. The standard errors of the mean for all datum points were less than 5% (A), 7% (B), and 13% (C). Net target cell lysis is shown for 76 injected mice, all macaques (six), and all baboons (four) that have been vector-immunized (D). Each dot represents net target cell lysis at an effector/target ratio of 100:1 for individual mice, macaques, and baboons. The peak CTL response for nonhuman primates is depicted, as these animals were tested at various time points postimmunization.

targets (BC-env/rev; $H\text{-}2^d$) but not allogeneic target cells expressing HIV-1 env and rev proteins (BL6-env/rev; $H\text{-}2^b$) (Table 2), indicating that the mouse CTL were MHC restricted. In subsequent mouse experiments, human target cells (HT1080) transfected with the murine class I $H\text{-}2D^d$ gene and infected with a recombinant vaccinia virus expressing HIV-1 gp160 were specifically lysed by vector-induced mouse CTL (data not shown). These data confirm that the mouse effector cells were restricted to the $H\text{-}2D^d$ MHC molecule. In preliminary experiments, the CTL from macaques and baboons lysed autologous target cells but were unable to lyse heterologous macaque and baboon target cells, respectively, suggesting that these cytotoxic responses were also self-MHC restricted (Table 2). Therefore, conventional class I MHC-restricted, $CD8^+$ CTL were generated in mice and nonhuman primates as a result of the direct administration of the N2 IIIBenv retroviral vector.

Examination of CTL induction in BALB/c mice with respect to vector dose demonstrated a threshold relationship. By injecting mice with serial dilutions of the N2 IIIBenv vector ranging from 8×10^5 to 9×10^4 CFU, splenocyte effectors that caused equivalent target cell lysis were generated (Fig. 3). Mice injected with a smaller quantity of vector (3×10^4 or 1×10^4 CFU) exhibited negligible target cell lysis. These studies were extended by injecting groups of mice (five to seven) with a single batch of vector that was diluted to 10^7, 10^6, 10^5, and 10^4 CFU/ml and confirmed by titer assay. All mice injected with 10^7 and 10^6 CFU/ml (seven of seven and five of five, respectively) generated CTL responses (greater than 15% net lysis at an effector/target ratio of 100:1). Two of six mice injected with 10^5 CFU/ml generated CTL responses of 31 and 43% specific net lysis, and no mice injected with 10^4 CFU/ml exhibited CTL responses. Several other experiments, involving injection of different preparations of the N2 IIIBenv vector, demonstrated a similar threshold phenomenon for CTL induction. Analysis of these data indicates that, in mice, a titer of approximately 10^5 CFU/ml was necessary for CTL induction.

The induction of an immune response by recombinant

TABLE 1. CTL responses from rhesus macaques and baboons injected with N2 IIIB env

Animal[a]	% Specific net target cell lysis[b] (wk)					
	Wk 0	Wk 2	Wk 2–4	Wk 6	Wk 16	Extended
Macaques						
R168	3		56		47	38 (22), ND
R178	0		14		60	28 (18), 9 (20)
R180	0		7		30	33 (26), 6 (30)
R188	1		50		34 (28)	14 (32), 9 (36)
R190	4		40		34	ND, ND
R156	4		2		30	8 (25), 7 (27)
Baboons						
B36	7	22		45		11 (10)
B38	6	16		18		4 (10)
B40	0	17		12		38 (18)
B42	0	17		34		45 (14)

[a] Times of injection (weeks): R168, 0, 2, 4, 16, and 18; R178, 0, 12, 14, and 16; R180, 0, 4, 8, 12, 18, and 22; R188, 0; R190, 0, 4, 8, and 12; R156, 0, 2, 4, 10, 12, 14, 23, and 25; B36 and B38, 0 and 4; B40 and B42, 0, 2, and 4.

[b] Lysis of HIV-1 env/rev-expressing autologous targets − lysis of autologous herpesvirus papio-transformed targets at an effector-to-target cell ratio of 100:1. Effector CTL were obtained from peripheral blood lymphocytes at the indicated sampling times before (week 0) and after administration of N2 IIIB env. ND, not done.

retroviral vectors could be mediated by transduction and de novo protein synthesis or, potentially, by foreign vector-associated proteins. To determine whether the observed CTL responses were dependent upon N2 IIIBenv-mediated in vivo transduction or the potential presence of soluble HIV-1 proteins contained in the vector preparation could stimulate CTL, vector particles were inactivated prior to immunization. The vector was exposed to either Nonidet P-40 (0.05%) or heat (68°C, 30 min) treatment so as to not denature proteins present in the vector preparation but to inactivate transduction by the vector. Treated N2 IIIBenv and untreated control vectors were titered and administered to mice to evaluate CTL induction. These treatments did inactivate the vector, as the titers were substantially reduced ($<10^4$ CFU/ml) compared with the untreated control vector (8×10^5 CFU/ml). Neither the Nonidet P-40-treated nor the heat-treated vector generated CTL activity (0% net target cell lysis at 100:1), whereas the untreated vector induced HIV-1-specific CTL activity (52 and 54% net target cell lysis at 100:1). These results suggest that transduction of host cells by the recombinant retroviral vector is essential for generating the observed CTL responses.

The N2 IIIBenv vector encodes different proteins (env, rev, and Neo^r). Therefore, N2 IIIBenv-induced CTL were tested on various target cells to evaluate the specificity of the vector-induced CTL response. CTL lines, generated by priming mice with the N2 IIIBenv vector and restimulating in vitro with irradiated BC-env/rev cells, were tested on targets expressing individual HIV-1 proteins. The lack of lysis of BC-βgal targets compared with BC-env/rev targets indicated that the cytotoxic activity was specific for vector-encoded HIV-1 proteins and not an irrelevant protein or the Neo^r marker, which is shared by both target cells (Fig. 1A and Table 3). The HIV-1 env protein is produced in the absence of the rev protein in target cells infected with a recombinant vaccinia virus that encodes HIV-1 IIIB gp160 (Vac-gp160) (6). N2 IIIBenv-induced CTL lines recognized and lysed BC target cells infected with Vac-gp160 (BC+Vac-gp160) but not targets infected with a control recombinant vaccinia virus expressing the bacterial LacZ gene (BC+Vac-LacZ) (6), indicating that

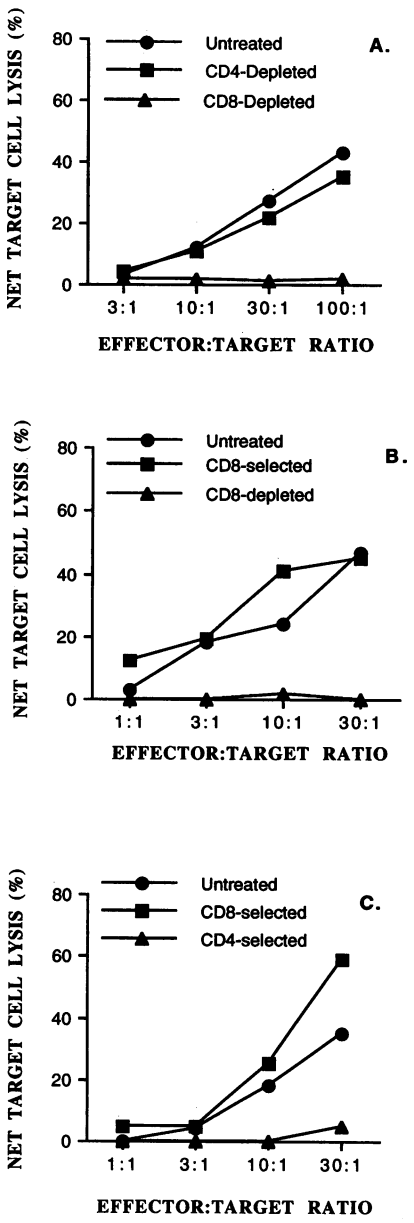


FIG. 2. Cytolytic effector cells generated by N2 IIIBenv immunization are CD8$^+$ T cells. Cytolytic effector cells from animals injected with the N2 IIIBenv vector were treated with specific antibodies and separated into CD8-enriched (CD8$^+$) or CD8-depleted (CD4$^+$) cell populations. Control effector cell populations were not antibody treated (untreated). The net of the mean of specific target cell lysis is plotted for mouse (A), macaque (B), and baboon (C) effector cells at the indicated effector-to-target cell ratios.

HIV-1 env-specific CTL were present in the polyclonal effector cell population. To investigate whether these CTL lines also contained rev-specific effector cells, target cells transduced with a retroviral vector encoding only HIV-1 rev (BC-rev) were employed. N2 IIIBenv-induced CTL lines showed rev-specific target cell lysis, as BC-rev targets and BC-env/rev targets were lysed while BC-βgal control targets were not (Table 3). Therefore, N2 IIIBenv vector administration to BALB/c mice generated CTL responses specific for both env and rev protein epitopes.

TABLE 2. MHC restriction of N2 IIIBenv-induced CTL[a]

Animal and E/T	% Net target cell lysis	
	Syngeneic or autologous	Allogeneic or heterologous
Mouse		
100:1	79	0
30:1	60	2
10:1	35	1
3:1	14	2
Macaque R168		
25:1	28	0
8:1	19	0
3:1	11	0
1:1	2	0
Baboon B42		
100:1	45	4
30:1	35	2
10:1	18	2
3:1	4	0

[a] CTL were tested for lysis of MHC-compatible and -incompatible target cells expressing HIV-1 env/rev. Vector-induced BALB/c ($H\text{-}2^d$) CTL were tested for lysis of syngeneic (BC-env/rev; $H\text{-}2^d$) and allogeneic (BL/6-env/rev; $H\text{-}2^b$) targets at the indicated effector/target (E/T) ratios. Rhesus macaque and baboon vector-induced CTL were tested for lysis of autologous and heterologous HIV-1 env/rev-expressing targets.

To further characterize the env-specific component of the mouse CTL response, a peptide (p18 IIIB) corresponding to the HIV-1 IIIB hypervariable V3 loop region of the env protein was used to pulse-label target cells. This peptide is an immunodominant CTL epitope, recognized by BALB/c mouse T cells specific for the HIV-1 IIIB env protein (35). Target cells pulsed with this peptide (BC+p18 IIIB) were recognized and lysed by N2 IIIBenv-induced CTL lines, in contrast to control BC targets (Fig. 4). Direct N2 IIIBenv vector administration to

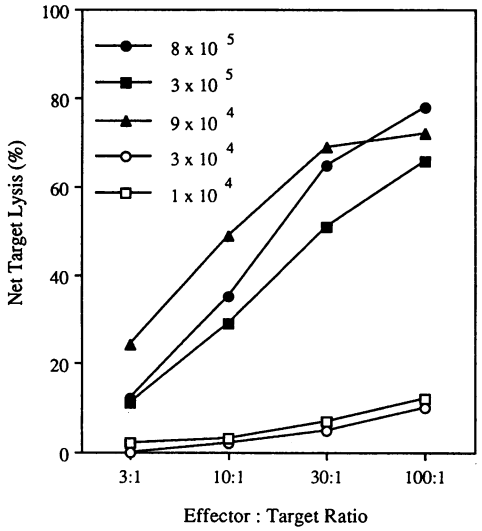


FIG. 3. Mice injected with serial dilutions of N2 IIIBenv exhibit a threshold response for CTL induction. Mice were immunized with serial dilutions of the N2 IIIBenv vector and tested for antigen-specific CTL induction. The mean of the net percent target cell lysis is plotted for effector cells generated with the indicated vector doses (CFU) and at the indicated effector-to-target cell ratios. The standard error of the mean for triplicate assay wells was less than 3% at each datum point.

TABLE 3. HIV-1 IIIB env and rev specificities of N2 IIIBenv-induced CTL[a]

E/T[b]	% Lysis of target cells expressing:				
	BC-env/rev	BC-βgal	BC-rev	BC+Vac-gp160	BC+Vac-LacZ
30:1	68	0	44	62	9
10:1	45	0	20	44	4
3:1	18	0	8	12	0

[a] CTL lines were generated from mice that were injected with the N2 IIIBenv vector. The BC-env/rev target cell expresses the HIV-1 env and rev proteins from HIV-1IIIB, whereas the BC-rev target cell expresses only the rev protein. The BC-βgal target cell expresses bacterial β-galactosidase. BC10ME target cells were also infected with recombinant vaccinia viruses encoding the HIV-1 IIIB gp160 gene but not the rev gene (BC+Vac-gp160) or the bacterial LacZ gene (BC+Vac-LacZ).

[b] E/T, effector/target ratio.

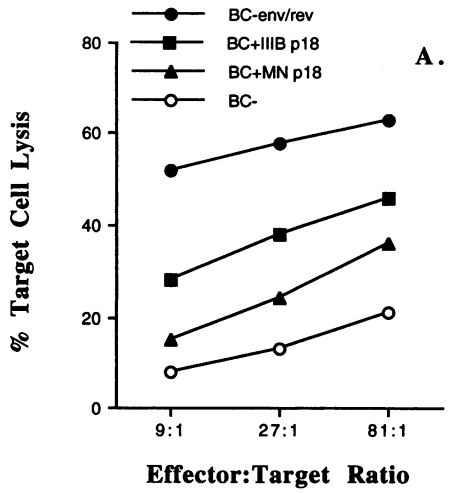
mice induced a cross-reactive CTL response in two of five CTL lines, as demonstrated by lysis of targets coated with an equivalent peptide derived from the V3 loop region of the HIV-1 MN viral isolate (Fig. 4). These results suggested that the vector-induced (HIV-1 IIIB env-specific) CTL population was capable of recognizing cross-reactive determinants of an env protein from a different HIV-1 isolate.

HIV-1 env-specific antibody responses were also examined for mice and nonhuman primates immunized with the N2 IIIBenv retroviral vector. Serum samples obtained from animals prior to and after injection of N2 IIIBenv were tested by ELISA for HIV-1 gp120-specific antibody. High-titer antibody responses were observed in six BALB/c mice after the second administration of vector and persisted for at least 12 months after the third injection (Fig. 5A). Preinjection mouse sera showed no anti-gp120 specific antibody, nor did sera collected after the first injection. Sera from one of four vector-immunized baboons have shown gp120-specific antibody titers by ELISA (Fig. 5B), but rhesus macaque sera have, thus far, been negative for anti-gp120 reactivity (data not shown). More recent attempts at generating antibody responses in mice injected with N2 IIIBenv have not been successful. Interestingly, vector preparations that successfully induced antibody in mice were supernatants from producer cells, which possess quantities of soluble env protein detectable by Western blot analysis. Current vector preparations which have failed to elicit antibody responses contain undetectable quantities of soluble env protein, by Western blot analysis. We are currently investigating particular vector compositions capable of inducing antibody responses.

DISCUSSION

Retrovirus-mediated gene transfer is an efficient means of delivering genes encoding foreign proteins. Direct injection of the N2 IIIBenv retroviral vector leads to activation of class I MHC-restricted $CD8^+$ T-cell responses specific for the HIV-1 IIIB env and rev proteins encoded by the vector in each of the three animal models examined. CTL induction was shown to be dependent on the vector transduction process and was not the result of associated soluble gp160/120 protein. Induction of CTL memory cell activity was also apparent, because CTL responses have been observed at least 6 months after vector administration in mice and macaques and 4 months postinjection in a baboon. Therefore, the N2 IIIBenv retroviral vector appears to be quite capable of inducing long-lived CTL activity in the different animal models.

The CTL response in HIV-infected individuals likely con-



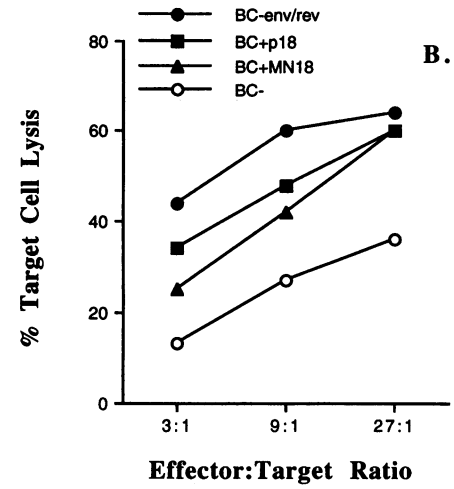
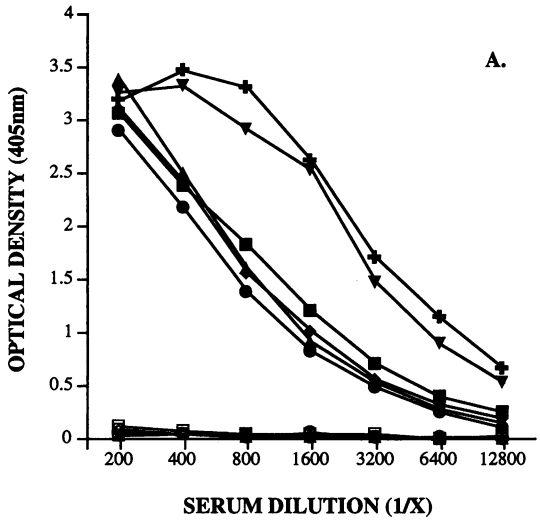


FIG. 4. N2 IIIBenv-induced CTL can recognize target cells coated with a peptide from the HIV-1 IIIB envelope protein and cross-react with target cells coated with the analogous peptide from HIV-1 MN. Two independently derived CTL lines (A and B) were generated by in vitro restimulation of N2 IIIBenv-primed splenocytes with BC-env/rev cells and recombinant interleukin 2. Effector cells were tested for recognition of BC10ME target cells (BC) or BC-env/rev target cells, which express the HIV-1 IIIB envelope and rev proteins. BC10ME target cells were also coated with peptides derived from the V3 loop region of either the HIV-1 envelope protein of the IIIB strain (BC+p18 IIIB) or the MN strain (BC+p18 MN). Standard error of the mean for triplicate assay wells was less than 3% for each datum point.



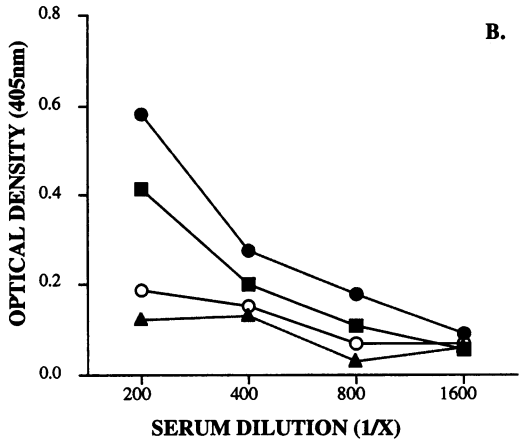


FIG. 5. HIV-1 envelope gp120-specific antibody is generated in mice and baboons by administration of N2 IIIBenv. (A) BALB/c mice were injected i.m. (0.1 ml per muscle site) into each hind limb three times with the N2 IIIBenv vector (1.6×10^6 CFU/ml). Open symbols, serum antibody responses specific for gp120 prior to immunization; solid symbols, postimmunization serum antibody responses for each individual mouse. (B) Baboons were injected i.m. with 3 ml of N2 IIIB env (1.0×10^7 CFU/ml) distributed to six muscle sites every 2 weeks for a total of three injections. Serum samples were collected from all animals prior to each injection and tested by ELISA for the presence of HIV-1 envelope gp120-specific antibody. Serum antibody responses from one baboon prior to injection (○) and after the first (▲), second (■), and third (●) injections are shown. Serum samples from three other immunized baboons have failed to generate detectable antibody responses.

tributes to maintenance of the asymptomatic phase of the disease (5). Current approaches to enhance HIV-specific immunity in HIV-infected individuals have included administering soluble recombinant HIV-1 gp160/120 protein (10, 15). These treatments have shown increased $CD8^+$ and $CD4^+$ CTL responses as well as antibody responses. $CD4^+$ CTL, in contrast to $CD8^+$ CTL, have been shown in vitro to lyse non-HIV-infected targets that have surface-bound HIV-1 gp120 protein (24, 33). $CD4^+$ CTL lysis of gp120-bound $CD4^+$ T cells could result in the elimination of non-HIV-infected target cells in vivo, an argument invoked to possibly explain the loss of $CD4^+$ T cells during HIV infection. Conversely, $CD8^+$ CTL appear to specifically recognize virally infected cells and do not exhibit lysis of targets that are exogenously protein coated. Therefore, specific induction of $CD8^+$ CTL may be preferred to $CD4^+$ CTL induction in the HIV infection and disease process.

The ability to induce broad reactivity against different HIV

isolates is an essential component of potential HIV vaccines and immunotherapeutics. The HIV-1 env/rev-specific CTL generated in some mice by administration of N2 IIIBenv exhibited cross-reactivity on targets coated with an immunodominant env peptide derived from the V3 hypervariable region of the HIV-1 IIIB and HIV-1 MN env proteins (36). Previous studies employing immunization with ex-vivo vector-transduced autologous fibroblasts have generated similar V3 peptide cross-reactive CTL (41). A more comprehensive analysis, employing target cells infected with different prototypic and clinical HIV-1 isolates demonstrated extensive cross-strain recognition and lysis with CTL induced by injection of N2 IIIBenv-transduced syngeneic fibroblasts (6). CTL recognition of common HIV-1 determinants by N2 IIIBenv vector-induced CTL could be important in eliminating cells infected with diverse HIV isolates as well as variant, sequential intrapatient viruses.

CTL specific for the HIV-1 rev protein have been shown following N2 IIIBenv immunization of BALB/c mice. Also, preliminary evidence suggests that the baboon CTL response contains effectors directed at a peptide epitope derived from the rev protein (data not shown). The CTL response specific for rev determinants may be of particular importance. Firstly, the rev protein is expressed early in virus infection, and lysis of virus-infected cells prior to mature virion production could be beneficial in controlling virus spread. Secondly, CTL specific for rev determinants may also be cross-reactive with cells infected with different HIV-1 isolates because the rev protein appears to be conserved (21). Immune responses directed at conserved determinants are likely to provide the broadest specificity and, thus, be effective over the prolonged time course typically seen in HIV infections.

HIV-1 antibody responses are likely important for clearing free virus. HIV-1 env-specific antibody responses can lead to direct virus neutralization or could contribute to antibody-dependent cellular cytotoxicity (30). The gp120-specific antibody response generated in vector-injected mice indicates that humoral immunity can also be involved in the immune response induced by the N2 IIIBenv vector. High-titer serum antibody, specific for gp120, persisted in N2 IIIBenv vector-treated animals for at least 1 year after immunization (data not shown), suggesting potential long-term gene expression in these animals. Studies are under way to determine the longevity of integrated vector DNA as a potential explanation for the persistence of env-specific antibody.

The vector preparation used in the mouse antibody studies (Fig. 5A) was a filtered vector supernatant and contained soluble proteins including fetal bovine serum and vector-generated HIV envelope protein. Current vector preparations are column purified and have markedly reduced free-protein levels. Interestingly, envelope-specific antibody responses in mice and macaques to these vector preparations are undetectable. An HIV-1-specific antibody response in one of the four baboons injected with purified vector has been detected (Fig. 5B), but the titer was relatively low and other baboon immune serum samples have been envelope-specific antibody negative. Rhesus macaques do generate substantial HIV-1-specific antibody responses following injection of 5×10^7 N2 IIIBenv-transduced autologous fibroblasts (17), as do similarly treated mice (41). The induction of an antibody response probably requires more soluble envelope protein than that provided by N2 IIIBenv in vivo-transduced cells, under the present conditions. Studies are under way to determine under what conditions the present vector preparations can generate an antibody response. Furthermore, we are investigating which cell types have been transduced in vivo and which cells subsequently act as the antigen-presenting cells that drive envelope-specific immune responses. Induction of an immune response involving both cellular immunity and humoral immunity is likely to provide the greatest impact on the HIV disease process. Retrovector immunization may represent at least one approach for consistently activating these immune responses.

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