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Induction of Vigorous Cytotoxic T-Lymphocyte Responses by Live Attenuated Simian Immunodeficiency Virus

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Although live attenuated vaccine strains of simian immunodeficiency virus (SIV) have proven highly effective in protecting macaques against challenge with pathogenic SIV strains, little is known about the mechanisms of protective immunity induced by these vaccines. We examined cytotoxic T-lymphocyte (CTL) responses against SIV in animals infected with SIVmac239Δnef (deficient in *nef*) or SIVmac239Δ3 (deficient in *nef*, *vpr*, and upstream sequences in U3). To enhance detection of SIV-specific CTL activity, we stimulated peripheral blood mononuclear cells with autologous B-lymphoblastoid cell lines which had been infected with recombinant vaccinia viruses expressing SIV proteins and subsequently inactivated with psoralen and UV light. Animals chronically infected with SIV239Δnef or SIV239Δ3 mounted vigorous CTL responses against the SIV Gag and Env proteins. This CTL activity was major histocompatibility class restricted and mediated by $CD8^+$ T lymphocytes. CTL responses persisted at relatively high levels for more than 6 years after infection. Limiting dilution precursor frequency assays demonstrated that the frequency of SIV-specific CTLs was as high as 234 CTL precursors per 100,000 cells. Animals acutely infected with SIV239Δnef developed CTL activity by day 14 after infection, coincident with decreases in viral load. Animals acutely infected with SIV239Δ3 developed CTL responses within 4 weeks of infection. Thus, vaccination of juvenile or adult animals with SIV239Δnef or SIV239Δ3 results in the induction of a vigorous CTL response which arises early in the course of infection and persists for years after a single inoculation of virus.

Development of an effective human immunodeficiency virus (HIV) vaccine is proving to be an elusive goal. Immunization of HIV type 1 (HIV-1) seronegative subjects with recombinant subunit vaccines has resulted in the production of HIV-specific antibodies, but these antibodies have neutralized primary HIV isolates poorly or not at all (41). Only a subset of candidate HIV vaccines have been able to induce cytotoxic T-lymphocyte (CTL) responses, and these responses have generally been relatively weak or transient (11, 17). Although subunit HIV-1 vaccines have induced protection against infection with HIV-1 in chimpanzees (3, 4, 18), it is uncertain whether such vaccines would be effective in protecting people against HIV-1 infection with primary HIV-1 isolates, especially many months after vaccination.

The limited progress to date towards an AIDS vaccine has prompted a renewed search for alternative vaccine strategies and better information on the nature of protective immunity. In nonhuman primates, impressive protection against challenge with pathogenic viruses has been observed following vaccination with live attenuated simian immunodeficiency (SIV) strains, particularly those strains deficient in *nef*. Animals vaccinated with SIVmac239 with a deletion in the *nef* gene (SIV239Δnef) are protected against challenge with pathogenic SIVmac239 and high doses of the heterologous challenge virus SIVmac251 (12). Subsequent studies have demonstrated that an attenuated virus with deletions in the *nef*, *vpr*, and upstream sequences of the long terminal repeat (SIV239Δ3) is also able to induce protective immunity against challenge with SIVmac251 (61). Other attenuated SIV strains are able to induce protective immunity (1, 9, 24, 56), although some attenuated strains provide either partial or no protection (37, 40, 61). The ability of live attenuated SIV strains to induce protection appears to vary with the length of time after vaccination (9, 56, 61) and the degree of attenuation (37) and probably with other factors yet to be defined.

Although the feasibility of live attenuated HIV vaccines for use in humans remains controversial (2, 6, 15), analysis of macaques vaccinated with live attenuated SIV offers a valuable experimental model to investigate the mechanisms of protective immunity. Studies of uninfected individuals exposed to HIV-1 have not yielded unequivocal evidence for immune responses that are able to protect against infection. Although a subset of exposed uninfected individuals possess HIV-specific proliferative (10) and CTL responses (14, 34, 49, 53), it is not clear whether these responses represent the immunologic hallmark of an abortive infection or the immune responses responsible for preventing infection. In fact, recent evidence suggests that a mutation in the CCR5 coreceptor for primary HIV-1 isolates is likely to be responsible for resistance to infection in at least some exposed uninfected individuals (36, 48). In contrast, macaques vaccinated with SIV239Δnef or SIV239Δ3 are clearly protected from infection, thus permitting investigation of the immune responses that contribute to protection. Recent data from a vaccine trial with SIV239Δ3 have provided some clues as to the nature of protective immunity induced by live attenuated SIV (61). A notable characteristic of immunity induced by SIV239Δ3 in this report was that it appeared to be relatively delayed in onset, taking between 20 and 79 weeks to

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reach maximum protection (61). Analysis of neutralizing antibody responses in these animals revealed that neutralization of a primary SIV challenge stock, but not a laboratory-adapted SIV strain, correlated with protection. However, whether neutralizing antibodies play a direct role in protection is not known, and no information on SIV-specific CTL responses in animals vaccinated with SIV239Δnef or SIV239Δ3 is currently available.

In this study, we examined SIV-specific CTL responses in animals vaccinated with SIV239Δnef or SIV239Δ3. Following in vitro stimulation with autologous cells expressing SIV antigens, we analyzed CTL activity in animals vaccinated with these attenuated SIV strains at different time points after infection. Our results show that vaccination with SIV239Δnef or SIV239Δ3 results in the induction of a vigorous major histocompatibility complex (MHC) class I-restricted CTL response that developed in the first 2 to 4 weeks of infection and persisted for at least 6 years.

MATERIALS AND METHODS

Animals. Rhesus macaques used in the study were housed either at the New England Regional Primate Research Center or the Yerkes Regional Primate Research Center. Animals were infected intravenously with the attenuated strains SIVmac239Δnef (32) or SIVmac239Δ3 (deficient in *nef*, *vpr*, and the negative regulatory elements of the long terminal repeat [61]) or with a pathogenic molecular clone of SIV (SIVmac239) (32).

Animals were maintained in accordance with the guidelines of the local institutional animal use committees and of the federal government (45a).

Cell lines. Autologous transformed B-lymphoblastoid cell lines (B-LCL) from study animals were transformed by incubating peripheral blood mononuclear cells (PBMC) at 37°C with herpesvirus papio derived from the supernatant of S594 cells (provided by N. Letvin, Beth Israel Hospital, Boston, Mass.) and cyclosporin (1 μg/ml) in RPMI 1640 (Cellgro) with 20% fetal bovine serum (FBS). B-LCL were propagated in RPMI 1640 supplemented with 20% FBS, 10 mM HEPES, 2 mM L-glutamine, 50 IU of penicillin/ml, and 50 μg of streptomycin/ml.

Stimulation of effector cells for CTL assays. PBMC were isolated from fresh heparinized blood by centrifugation over a Ficoll-sodium diatrizoate gradient (Ficoll 1077; Sigma, St. Louis, Mo.) and suspended at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 50 IU of penicillin/ml, and 50 μg of streptomycin/ml (R10 medium). For lectin stimulation, PBMC were incubated with concanavalin A (ConA; Sigma) at a concentration of 5 μg/ml for 3 days, washed, and then resuspended in R10 medium supplemented with 10 to 20 U of recombinant human interleukin-2 (IL-2) (donated by M. Gately, Hoffman LaRoche). For antigen-specific stimulation, B-LCL were infected at a multiplicity of infection of 3 to 10 PFU/cell with a recombinant vaccinia virus (vAbt388, provided by D. Panicali, Therion Biologics, Cambridge, Mass.) containing the SIVmac251 *gag* and *pol* genes and the SIVmac239 *env* gene. After an overnight incubation, infected B-LCL cells were inactivated with long-wave UV irradiation (Fisher Model UV, 350- to 400-nm wavelength) in the presence of 10 μg of psoralen (HRI Associates)/ml. Cells were UV irradiated for 10 min at a distance of 3.5 cm from the light source, washed three times, and then used as stimulators. PBMC were then cultured with stimulator cells at a responder to stimulator ratio of 10:1 in R10 medium. Recombinant IL-2 was added to a final concentration of 10 to 20 U/ml on day 4 of culture. CTL assays were performed 10 to 14 days after stimulation.

Chromium release assay. Target cells consisted of autologous or allogeneic B-LCL infected with recombinant vaccinia viruses expressing SIV proteins. Allogeneic B-LCL were selected so as to minimize class I homology as assessed by one-dimensional isoelectric focusing of class I MHC immunoprecipitates (30, 60). Recombinant vaccinia viruses used to infect target cells included vAbt252 (encoding the SIVmac251 p55 gag and protease proteins; Therion), rVV-239 (encoding the SIVmac239 envelope [Env]) (52), and the control vaccinia virus NYCBH. B-LCL were infected overnight with a multiplicity of infection of 5 to 10 PFU/cell and then labeled with 100 μCi of ^{51}chromium (DuPont NEN, Wilmington, Del.) per 10^6 cells. Target cells (10^4 cells/well) were dispensed in duplicate for each effector/target (E/T) ratio into 96-well U-bottomed plates (Costar). For most assays, cold target inhibition was used to decrease background lysis. Cold targets consisted of unlabeled autologous B-LCL that had been infected with the control vaccinia virus NYCBH and were used at a cold target/hot target ratio of 15:1. Chromium release was assayed after a 5-h incubation at 37°C in a 5% CO_2 incubator. Plates were spun at 1,000 rpm for 10 min at 4°C, after which 30 μl of supernatant was harvested from each well into wells of a LumaPlate-96 (Packard) and allowed to dry overnight. Emitted radioactivity was measured in a 1450 MicroBeta Plus liquid scintillation counter (Wallac, Turku, Finland). Spontaneous release was measured from wells containing only target cells and medium. Maximum release was measured from wells containing target cells and 0.1% Triton X-100 (Sigma). The percent specific cytotoxicity was calculated as follows: (test release − spontaneous release)/(maximum release − spontaneous release) × 100. Spontaneous release of target cells was <25% in all assays. E/T ratios for which background lysis of control targets exceeded 20% were excluded from analysis. Based on examination of control animals not infected with SIV, SIV-specific lysis of greater than 5% seen at more than one E/T ratio was interpreted as significant.

CD8$^+$ and CD4$^+$ lymphocyte separation. CD8$^+$ lymphocytes were prepared from stimulated PBMC by depletion of CD4$^+$ cells with an anti-CD4 monoclonal antibody (OKT4) at 20 μg/10^6 cells and then incubation with anti-mouse immunoglobulin G magnetic beads (Perseptive Diagnostics, Framingham, Mass.) at a 50:1 bead to cell ratio for 30 min at 4°C. The supernatant enriched for CD8$^+$ cells was collected by using a magnetic separation device (Dynal, Oslo, Norway). Similarly, the CD4$^+$ lymphocytes were obtained by coating cells with a CD8-specific monoclonal antibody (51.1, ATCC catalog number HB230) at a concentration of 20 μg/10^6 cells and then incubating them with anti-immunoglobulin G2a magnetic beads (Dynal) at a 10:1 bead to cell ratio for 60 min at 4°C. CD4$^+$-enriched cells in the supernatant were collected by using a magnetic separation device (Dynal). CD8$^+$ cell populations were greater than 80% CD8$^+$ and contained less than 9% CD4$^+$ cells. CD4$^+$ lymphocytes were greater than 90% CD4$^+$, as assessed by flow cytometry, with less than 1% CD8$^+$ cells.

Limiting dilution precursor frequency analysis of SIV-specific CTLs. To quantitate CTL responses, we used a modification of a limiting dilution precursor frequency assay previously described for the detection of HIV-1-specific CTLs (29). Fresh PBMC were seeded at 500 to 25,000 cells per well in 24 replicate wells of 96-well microtiter plates in the presence of 100 μl of R10 medium supplemented with recombinant IL-2 to a final concentration of 100 U per ml. A total of 2.5×10^4 gamma-irradiated (3,000 rads) human PBMC, with 3×10^4 to 4×10^4 autologous B-LCL infected with the recombinant vaccinia virus vAbt388 expressing the SIV *gag*, *pol*, and *env* genes, were added to each well. Prior to use, vaccinia virus-infected B-LCL were inactivated with psoralen-UV light as described above. On day 14 wells were split and tested for SIV-specific cytolytic activity by using as targets autologous ^{51}Cr-labeled B-LCL infected with either recombinant vaccinia viruses expressing SIV genes or a control vaccinia virus. For some assays, we used cold target inhibition to reduce background activity, adding unlabeled autologous B-LCL infected with control vaccinia virus (cold targets) to each well at a ratio of 10:1 cold to hot B-LCL. Wells for which lysis exceeded 10% lysis were scored as positive. The fraction of nonresponding wells was calculated for each dilution, and the precursor cell frequency was calculated by the maximum likelihood method (35) by using software developed by S. A. Kalams, Massachusetts General Hospital.

Virus isolation and SIV-specific antibody analysis. Peripheral blood from animals acutely infected with SIV239Δnef was collected for viral isolation prior to inoculation and at days 3, 7, 14, 21, 35, and 50 after inoculation. Quantitative viral cultures were performed as described previously (20). Briefly, serial threefold dilutions were performed in duplicate beginning with 10^6 PBMC. PBMC dilutions were cocultured with 10^5 CEM×174 cells in a volume of 1 ml. Cultures were split 1:2 twice weekly until day 21, when the cultures were assayed for virus production by SIV p27 by enzyme immunoassay (Coulter Corp., Miami, Fla.). Results are expressed as the number of SIV culture-positive cells/10^6 PBMC. SIV-specific antibodies in serum were detected by enzyme-linked immunosorbent assay (ELISA) with purified whole SIVmac as described previously (13).

RESULTS

Antigen-specific stimulation enhances detection of SIV-specific CTL activity. Detection of virus-specific CTL activity typically requires in vitro stimulation with autologous cells expressing processed viral antigens (65). Notable exceptions to the requirement for antigen-specific stimulation are the viruses HIV-1, SIV, and HTLV-I, for which CTL activity can be detected with fresh unstimulated PBMC (27, 28, 47, 59) or lectin-stimulated cells (45, 46). The ability to detect HIV- and SIV-specific CTLs without antigen-specific stimulation presumably reflects both the high level of circulating CTLs and the in vivo activation of CTL precursors to effector CTLs as a result of ongoing viral replication. Because stabilized viral loads in juvenile or adult macaques chronically infected with SIV239Δnef or SIV239Δ3 are at least 100-fold lower than in wild-type-infected animals (32, 61), we anticipated that existing techniques that do not utilize antigen-specific stimulation for detection of SIV-specific CTLs would be relatively insensitive for detection of CTL activity in animals infected with attenuated retroviruses (37). We therefore utilized autologous herpesvirus papio-transformed B-LCL expressing SIV proteins to stimulate SIV-specific CTLs, adapted after techniques previously

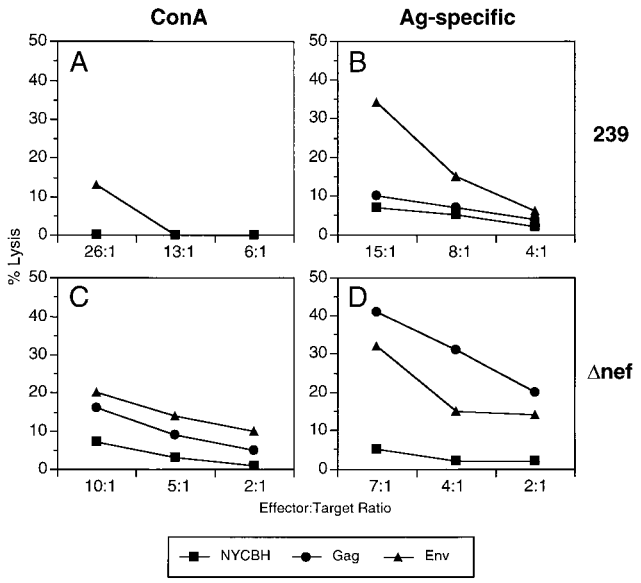

FIG. 1. Enhanced SIV-specific CTL activity following antigen-specific stimulation. PBMC from animals infected with either SIVmac239 (239) or SIVmac239Δnef (Δnef) were stimulated with either ConA or autologous B-LCL infected with a recombinant vaccinia virus expressing *gag*, *pol*, and *env* and inactivated with UV-psoralen treatment (Ag-specific). Effectors were tested for SIV-specific CTL activity against autologous target cells infected with either the NYCBH control or recombinant vaccinia virus expressing either *gag* or *env* after 10 days of culture.

described to enhance detection of HIV-specific CTLs (25, 39). Autologous B-LCL were infected overnight with a recombinant vaccinia virus expressing the SIVmac *gag*, *pol*, and *env* genes and then treated with psoralen and UV irradiation to inactivate the vaccinia virus vector. Effector cells from an animal infected with the pathogenic virus SIVmac239 had enhanced SIV-specific CTL activity following antigen-specific stimulation compared with effector cells stimulated with the lectin ConA alone (Fig. 1A and B). Enhancement of CTL activity was also observed with PBMC from an animal infected with the attenuated virus SIV239Δnef (Fig. 1C and D). CTL activity observed following in vitro stimulation with UV-psoralen-treated B-LCL did not represent in vitro induction of primary CTL responses, since no CTL responses were observed following antigen-specific stimulation of PBMC from uninfected animals (Fig. 2N and O and data not shown).

Macaques infected with SIV239Δnef or SIV239Δ3 develop $CD8^+$, MHC-restricted CTLs against SIV Gag and Env. Using the antigen-specific stimulation technique described above, we analyzed SIV-specific CTL activity in macaques infected with either SIV239Δnef or SIV239Δ3. Initial studies were performed in animals that had been infected for at least 3 years. Stimulated CTL activity was detected in all animals analyzed (Fig. 2). Relatively vigorous levels of CTL activity were observed against Gag and Env, with as much as 40% specific lysis observed with effector to target ratios as low as 3:1. CTL activities against Gag and Env were observed in all animals, with no clear increased recognition of one protein over the other. This CTL activity was MHC restricted, as no significant lysis of allogeneic B-LCL was observed (Fig. 3A). Fractionation of stimulated effector cells revealed significant CTL activity only in the $CD8^+$ fraction (Fig. 3B), consistent with prior reports of SIV-specific CTL activity (45, 58). These SIV-specific CTL responses represented a relatively durable response as the animals infected with SIV239Δnef and SIV239Δ3 had been infected between 3 and 6 years prior to analysis (32). Several of these animals had been subsequently challenged with pathogenic SIV between 2 and 3 years after vaccination but were protected from challenge as assessed by quantitative viral cultures, PCR, and lack of disease progression (12). All of these animals maintained relatively vigorous levels of CTL activity (Fig. 2D to G). Comparable levels of SIV-specific CTL activity were observed in challenged and unchallenged SIV239Δnef-infected animals. For comparison, we also analyzed CTL activity following antigen-specific stimulation in animals infected with the pathogenic virus SIVmac239. Because SIV-specific CTL activity generally declines in animals with advanced disease (8), we analyzed SIVmac239-infected animals prior to the onset of significant CD4 depletion (defined as CD4 of $< 30\%$) that had maintained SIV-specific CTL activity. Levels of SIV-specific CTL activity in these four animals infected with SIVmac239 were either comparable to or less than those observed in animals infected with attenuated SIV strains (Fig. 1B and 2K to M).

Limiting dilution analysis reveals a high frequency of SIV-specific CTL precursors in macaques infected with SIV239Δnef or SIV239Δ3. To quantitate more precisely the SIV-specific CTL response in animals infected with SIV239Δnef and SIV239Δ3, we performed limiting dilution precursor frequency analysis of SIV-specific CTLs. PBMC were cultured in multiple replicate wells of various cell concentrations with stimulator cells consisting of UV-psoralen-inactivated B-LCL infected with a recombinant vaccinia virus expressing *gag*, *pol*, and *env*. After 10 to 14 days, stimulated effector cells from individual wells were tested for CTL activity against B-LCL expressing SIV antigens, and the frequencies of CTL precursors were calculated by maximum likelihood analysis. In two animals infected with SIV239Δnef, we observed SIV-specific CTL precursors at a frequency ranging from 60 to 234 CTL per 10^6 PBMC (Fig. 4). Similar data were obtained from an animal infected with SIV239Δ3, in which an SIV-specific CTL precursor frequency of 155 per 10^6 PBMC was demonstrated. Limiting dilution precursor frequency analysis of SIV-specific CTL responses in animals infected with pathogenic SIV strains often resulted in poor growth of cells in vitro and a failure to detect SIV-specific CTLs. In one animal infected with SIVmac239, we observed an SIV-specific CTL precursor frequency of 147 per 10^6 PBMC (data not shown).

SIV-specific CTLs arise early in the course of SIV239Δnef or SIV239Δ3 infection. The observation that development of full protective immunity to uncloned SIVmac251 challenge in animals vaccinated with SIV239Δ3 appeared to be delayed in onset (61), arising sometime between 20 and 79 weeks, raised the question as to when CTL activity in animals vaccinated with attenuated SIV strains developed. Because the studies described above were carried out in animals that had been infected for several years, we prospectively analyzed the kinetics of the SIV-specific CTL response in animals infected with SIV239Δnef or SIV239Δ3. Initial studies were conducted with two animals infected with SIV239Δnef in which we analyzed CTL responses, viral load, and the development of SIV-specific antibodies. In both animals, CTL activity was first detected 14 days after infection, increased 21 days after infection, and continued to rise gradually out to day 50 (Fig. 5). As has been observed for animals with pathogenic SIVmac251 (50), the onset of the SIV-specific CTL response coincided with a decline in viral load. Detection of virus-binding antibodies by ELISA did not occur until 3 to 5 weeks postinfection, following the onset of CTL responses and the drop in viral replication (Fig. 5).

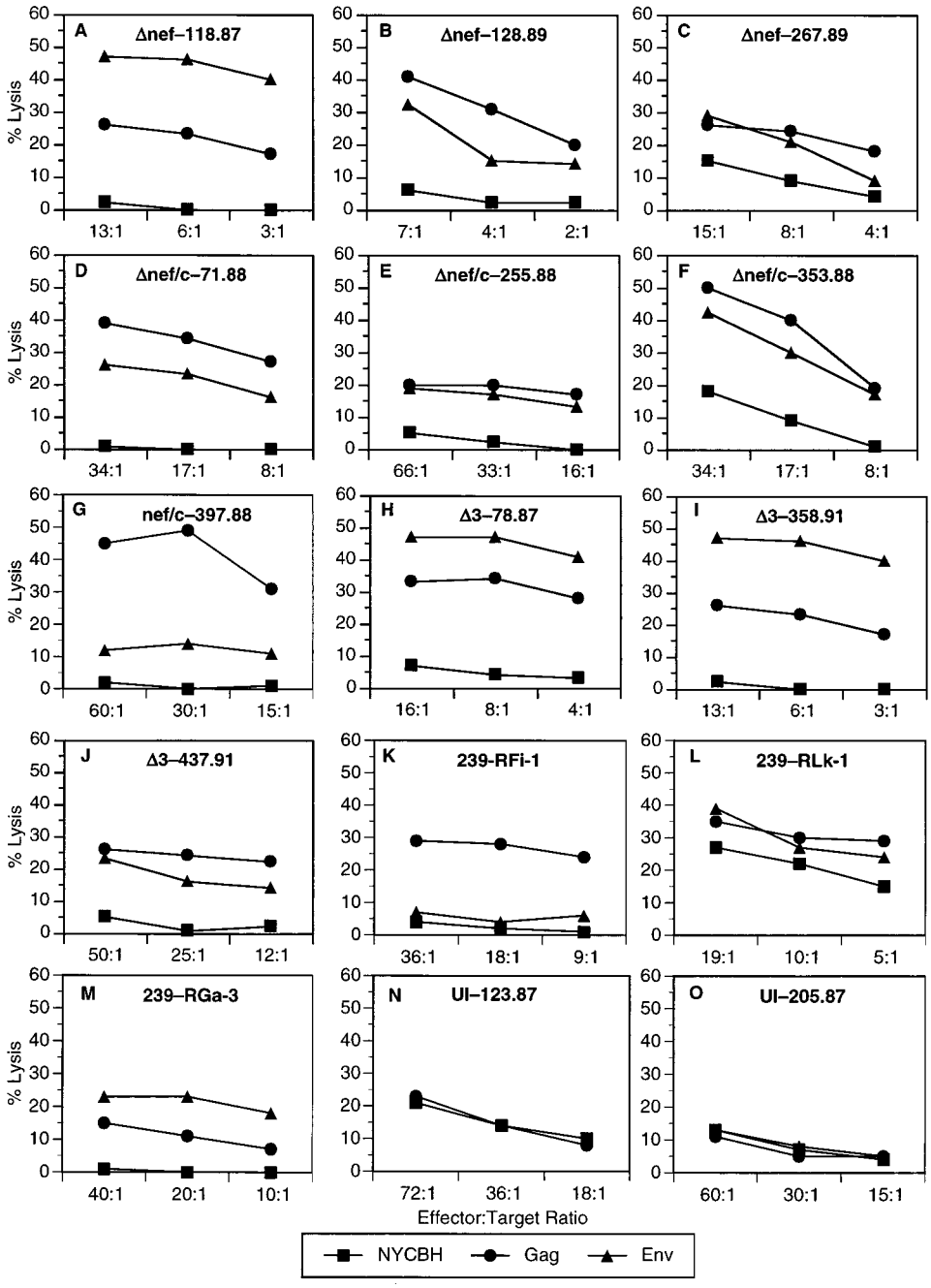

FIG. 2. SIV-specific CTL activity in macaques chronically infected with SIV239Δnef (Δnef) (A to C) or SIV239Δ3 (Δ3) (H to J). Results are also shown for animals subsequently challenged with pathogenic SIV strains (Δnef/c) (D-G) (all of which were protected from challenge ([12]), animals infected with SIVmac239 (239) (K to M), and uninfected controls (UI) (N and O). All effector cells were stimulated with autologous B-LCL infected with recombinant vaccinia virus expressing SIV proteins and inactivated with UV-psoralen treatment. E/T ratios are indicated below each figure.

We also prospectively analyzed the development of CTL activity in four animals infected with SIV239Δ3. SIV-specific CTL activity was detected in three of the four animals by 2 weeks after infection and in the remaining animal by 4 weeks after infection (Fig. 6).

DISCUSSION

The impressive ability of live attenuated SIV vaccines to induce protection against challenge with pathogenic strains of SIV has generated considerable speculation as to which mechanisms might be responsible for protection. As a first step in addressing the role of CTL responses in mediating protection, we analyzed stimulated CTL responses in animals vaccinated with SIV239Δnef or SIV239Δ3. Vaccinated animals developed relatively vigorous CTL activity against SIV Gag and Env. This CTL activity was mediated by classical $CD8^+$ MHC-restricted CTLs and was extremely durable, remaining detectable up to 6 years after initial vaccination. Despite the fact that viral loads in animals infected with SIV239Δnef or SIV239Δ3 are approx-

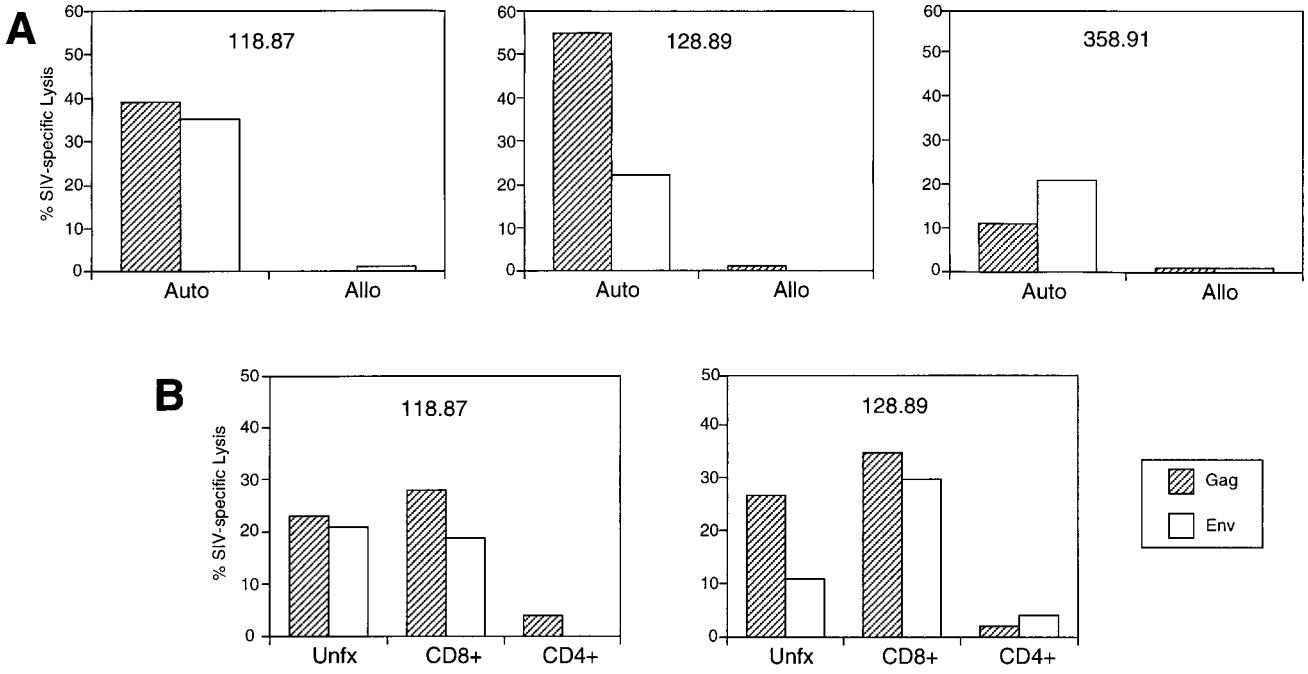

FIG. 3. SIV-specific CTL activity in macaques infected with attenuated SIV strains is MHC restricted and mediated by $CD8^+$ lymphocytes. (A) SIV-specific CTL activity in macaques infected with attenuated SIV strains is MHC restricted. Stimulated effector cells were analyzed for CTL activity against autologous (Auto) and MHC-mismatched (Allo) target cells. In order to minimize MHC class I homology of allogeneic B-LCL, MHC-mismatched B-LCL were selected based on one-dimensional isoelectric focusing of class I MHC immunoprecipitates. E/T ratios were 32:1 for 118.87, 10:1 for 128.89, and 8:1 for 358.91. (B) SIV-specific CTL activity in macaques infected with attenuated retroviruses is mediated by $CD8^+$ lymphocytes. Stimulated effector cells were separated with immunomagnetic beads into $CD8^+$ and $CD4^+$ populations. E/T ratios were 6:1 for 118.87 and 8:1 for 128.89. Unfx, unfractionated effector cells.

imately 100- to 1,000-fold lower than those in animals infected with the parental virus (32, 61), CTL activity following antigen-specific stimulation in animals vaccinated with these attenuated SIV strains appears to be comparable to or greater than that of animals infected with pathogenic SIVmac239. In fact, since we excluded SIVmac239-infected animals with advanced disease, CTL activity in animals infected with SIV239Δnef or SIV239Δ3 may exceed that of unselected animals infected with SIVmac239. The combination of low virus loads, strong stimulated CTL responses, and no disease progression seen with

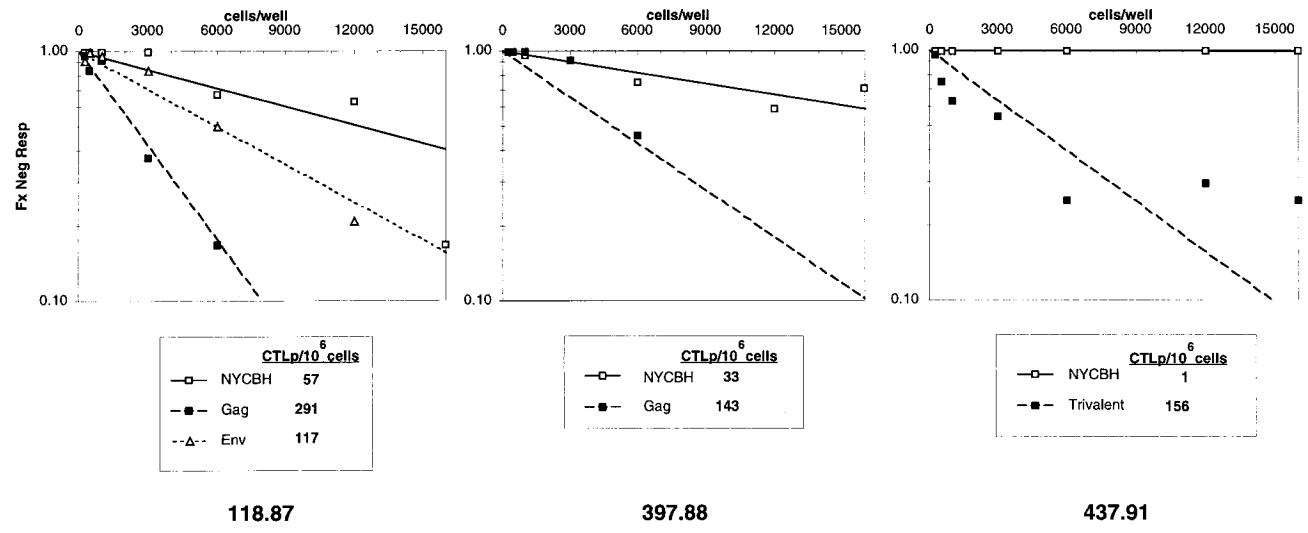

FIG. 4. Limiting dilution analysis of SIV-specific CTL precursors in animals infected with attenuated SIV strains. PBMC from animals infected with SIV239Δnef (118.87 and 397.88) or SIV239Δ3 (437.91) were stimulated with autologous B-LCL expressing SIV antigens, and then individual wells were tested for CTL activity after 10 to 14 days. The frequency of CTL precursors (CTLp) was calculated by maximum likelihood analysis and is shown as the frequency of CTLp per 10^6 PBMC. Data points for which all wells scored as positive (i.e., for which the fraction of negative responses was zero) are not plotted. The SIV trivalent vaccinia virus vector expresses the SIV *gag*, *pol*, and *env* genes. The unmodified NYCBH vaccinia virus was used as a negative control. Fx Neg Resp, fraction of negative responses.

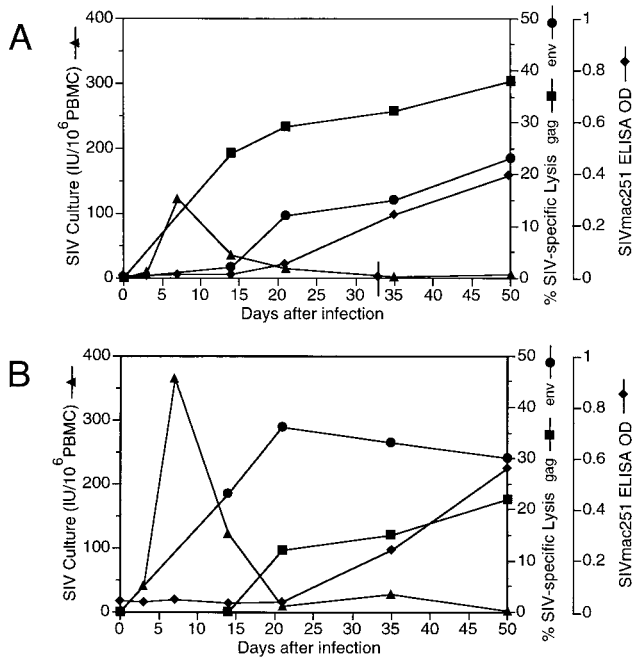

FIG. 5. Kinetics of SIV-specific CTL activity, antibody responses, and viral load in macaques acutely infected with SIV239Δnef. Viral load was determined by limiting dilution cultures and is expressed as infectious units (IU) per 10^6 PBMC. SIV-specific CTL activity was examined at multiple E/T ratios, and representative data are shown for E/T ratios ranging from 14:1 to 25:1. SIV-specific antibody levels were determined by ELISA with purified SIV, and values are plotted as optical density (OD) units.

the SIV vaccine strains in monkeys appears analogous in many ways to HIV-infected long-term nonprogressors, including one individual infected with *nef*-deleted HIV-1 (22, 23, 33, 51). Taken together, these observations support the conclusion that levels of stimulated SIV- or HIV-specific CTL activity are not necessarily proportional to viral load and that relatively vigorous CTL responses may be observed even with low viral loads.

A variety of vaccine approaches have been shown to induce $CD8^+$ CTL responses in macaques, including the use of peptides or modified peptides (7, 44), a recombinant *Mycobacterium bovis* BCG expressing SIV *gag* (62), recombinant vaccinia viruses expressing SIV *gag* (21, 54) or *nef* (19), and DNA immunization (38, 64). In light of the differing methodologies, it is difficult to quantitate precisely the relative strength of CTL responses induced by these different vaccine approaches. However, several observations suggest that the CTL responses induced by SIV239Δnef or SIV239Δ3 are comparable to or exceed those produced by other vaccines. Following a single in vitro stimulation, relatively high levels of lysis (>40%) were observed, even at E/T ratios less than 5:1. Although information on frequency of CTL precursors assessed by limiting dilution assays is only available for a subset of vaccine approaches, the level of CTL precursors we observed in animals vaccinated with SIV239Δnef or SIV239Δ3 is greater than that reported for other vaccines (19, 26, 31, 63) and is comparable to that observed in HIV-infected individuals by similar techniques (29, 55). However, technical differences in the limiting dilution assays employed may lead to different values for the numbers of CTL precursors. In addition, CTL responses induced by SIV239Δnef or SIV239Δ3 were detected in every animal examined in this study and persisted for over 6 years after initial infection. Immunization with the live attenuated SIV strain SIVmacC8, which expresses a mutant Nef protein, or with the attenuated strain SIVmac1A11 also results in $CD8^+$ CTL responses (16, 37). Although only limited data on the durability of CTL responses in vaccinated macaques are available, the ability of live attenuated SIV strains to induce long-lived CTL responses after a single inoculation is likely to be difficult to replicate with other vaccine approaches.

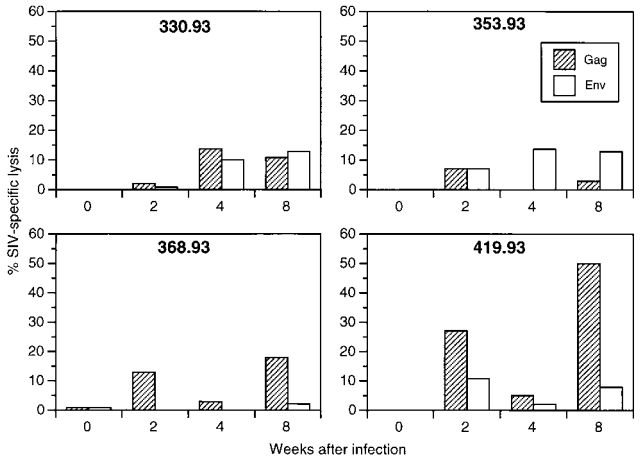

FIG. 6. Prospective analysis of SIV-specific CTL responses in macaques infected with SIV239Δ3. CTL activity was examined at multiple E/T ratios, and representative data are shown for an E/T ratio of 40:1.

The role that CTLs may play in mediating protective immunity induced by live attenuated SIV strains is difficult to assess at this time. Current experimental limitations of conducting research in nonhuman primates, such as the inability to achieve sustained depletion of T-cell subsets with monoclonal antibodies or to perform adoptive transfer of immune cells to naive animals, preclude a definitive answer to this question. However, several indirect observations support the hypothesis that CTLs may be involved in mediating protection. The presence of a durable and relatively vigorous SIV-specific CTL response in animals vaccinated with SIV239Δnef or SIV239Δ3, coupled with the expanding body of evidence supporting the conclusion that CTLs play an important role in controlling HIV-1 and SIV replication in vivo (reviewed in reference 42), suggests that CTLs may be involved in mediating protection. Further support for a role for CTLs in mediating protective immunity comes from the finding that SIV *nef*-specific CTLs induced by vaccination of macaques appear to be able to suppress viral replication after challenge or, in the case of one animal, prevent infection (19). Finally, macaques vaccinated with the attenuated pC8 strain (57) or with the attenuated SIVBK28 strain (5) are protected against infection with chimeric simian-human immunodeficiency viruses containing the HIV envelope. Similarly, animals immunized with a chimeric simian-human immunodeficiency virus containing the HIV envelope are resistant to vaginal challenge with SIVmac239 (43). Because neutralizing antibodies induced against SIV do not cross-neutralize the HIV envelope (and vice versa), such protection is likely to occur independently of neutralizing antibodies. However, the precise role of CTLs in mediating this protection remains to be defined.

Recent evidence suggests that the development of antibodies able to neutralize primary or heterologous isolates may correlate with the onset of protective immunity in animals vaccinated with live attenuated retroviruses. In the study by Wyand et al. (61), there was a strong, statistically significant correlation between the ability of serum to neutralize the pri-

mary challenge stock SIVmac251 and protection against challenge with the same stock. In contrast, no correlation with protection was observed for neutralization of laboratory-adapted SIVmac251. This observation appears to be consistent with the finding of Clements et al. who analyzed the correlation between humoral responses and protection in animals infected with the attenuated virus SIVmac17E-Cl (9). In SIVmac17E-Cl-infected animals, the onset of protection against challenge with the heterologous pathogenic virus SIVDeltaB670 approximately 8 months after infection correlated with the appearance of antibodies able to neutralize the challenge stock and with the development of SIV-specific envelope antibodies resistant to dissociation by 4 M urea. Taken together, these data suggest that the delayed onset of full protective immunity in animals infected with attenuated SIV strains may be associated with the maturation of the antibody response. However, since these animals infected with attenuated SIV strains are likely to have had SIV-specific CTL responses, this correlation does not exclude a potential role of CTLs in mediating protection.

Immune responses other than those of CTLs and neutralizing antibodies may also be involved in protective immunity. In particular, the potential role of soluble suppressor factors produced by $CD8^+$ lymphocytes from animals infected with attenuated SIV strains has yet to be elucidated. $CD8^+$ lymphocytes from animals infected with SIV239Δnef or SIV239Δ3 are able to produce soluble factors that inhibit SIV replication (19a), and characterization of this activity is in progress.

In summary, our studies demonstrate a vigorous SIV-specific CTL response in animals vaccinated with SIV239Δnef or SIV239Δ3. This CTL response is mediated by classical MHC-restricted $CD8^+$ CTLs and persisted for years after infection. Furthermore, attenuated SIV vaccine-induced CTLs were shown to be present at a relatively high CTL precursor frequency. Determination of the relative contributions of neutralizing antibodies and CTLs in mediating protection awaits the results of passive transfer experiments and the availability of experimental techniques to block or to adoptively transfer the activity of CTLs in vivo.

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