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A Vaccine-Elicited, Single Viral Epitope-Specific Cytotoxic T Lymphocyte Response Does Not Protect against Intravenous, Cell-Free Simian Immunodeficiency Virus Challenge

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Protection against simian immunodeficiency virus (SIV) challenge was assessed in rhesus monkeys with a vaccine-elicited, single SIV epitope-specific cytotoxic T-lymphocyte (CTL) response in the absence of SIV-specific antibody. Strategies were first explored for eliciting an optimal SIV Gag epitope-specific CTL response. These studies were performed in rhesus monkeys expressing the major histocompatibility complex (MHC) class I gene *Mamu-A*01*, a haplotype associated with a predominant SIV CTL epitope mapped to residues 182 to 190 of the Gag protein (p11C). We demonstrated that a combined modality immunization strategy using a recombinant *Mycobacterium bovis* BCG–SIV Gag construct for priming, and peptide formulated in liposome for boosting, elicited a greater p11C-specific CTL response than did a single immunization with peptide-liposome alone. Vaccinated and control monkeys were then challenged with cell-free SIV_{mne} by an intravenous route of inoculation. Despite a vigorous p11C-specific CTL response at the time of virus inoculation, all monkeys became infected with SIV. *gag* gene sequencing of the virus isolated from these monkeys demonstrated that the established viruses had no mutations in the p11C-coding region. Thus, the preexisting CTL response did not select for a viral variant that might escape T-cell immune recognition. These studies demonstrate that a potent SIV-specific CTL response can be elicited by combining live vector and peptide vaccine modalities. However, a single SIV Gag epitope-specific CTL response in the absence of SIV-specific antibody did not provide protection against a cell-free, intravenous SIV challenge.

Evidence is accumulating to support a central role for cytotoxic T lymphocytes (CTL) in clearing the viremia associated with an acute human immunodeficiency virus type 1 (HIV-1) infection. In a small number of carefully studied humans, a dramatic expansion of oligoclonal $CD8^+$ peripheral blood lymphocyte (PBL) populations during the primary phase of infection with HIV-1 has been described (16). Furthermore, clearance of the viremia associated with such a primary HIV-1 infection has been shown to occur coincident with the emergence of HIV-1-specific CTL and prior to the development of a detectable HIV-1 neutralizing antibody response (8). In a nonhuman primate model of HIV-1 infection, CTL are detectable as early as 4 to 6 days following virus inoculation, and virus clearance is associated temporally with the emergence of this CTL response (18, 30). It has been concluded from these observations that an effective HIV-1 vaccine should elicit HIV-1-specific CTL (9, 24).

The simian immunodeficiency virus (SIV)-macaque system has provided a powerful animal model for exploring vaccine strategies for the prevention of HIV-1 infections. SIVs have nucleotide sequence homology with HIVs and are tropic for $CD4^+$ lymphocytes and macrophages (2, 6). Moreover, selected SIV isolates induce an AIDS-like disease in macaques (10, 15). The SIV-macaque model has been used to assess traditional immunization strategies, including inactivated virus and live attenuated virus vaccines. It has also been used to explore more novel approaches to immunization, including live vector vaccines (9). The use of this model for developing AIDS vaccine strategies for eliciting CTL has been facilitated by the identification of SIV CTL epitopes and the major histocompatibility complex (MHC) class I molecules that bind viral peptide fragments and present them to CTL. We have used a well-defined predominant SIV Gag CTL epitope, residues 182 to 190 of the Gag protein, and the restricting rhesus monkey MHC class I molecule *Mamu-A*01* for exploring approaches to vaccine induction of CTL (14, 27).

We have shown previously that SIV Gag-specific CTL can be elicited in rhesus monkeys by both live vector approaches and peptide immunization (11, 20, 28, 29). In the present study, we explored a combined modality immunization strategy using a recombinant *Mycobacterium bovis* BCG-SIV Gag construct for priming and peptide formulated in a liposome for boosting, to optimize a SIV Gag-specific CTL response. We then assessed the protection against SIV challenge afforded by this vaccine-elicited SIV Gag-specific CTL response.

MATERIALS AND METHODS

Animals. Ten rhesus monkeys (*Macaca mulatta*) were used in these studies. Five (006, 007, 576, 036, and 637) expressed the MHC class I gene *Mamu-A*01*, as determined by one-dimensional isoelectric focusing studies (25). All animals were maintained in accordance with the guidelines of the Committee on Animals for Harvard Medical School and the *Guide for the Care and Use of Laboratory Animals* (23).

Vector construction and preparation of recombinant BCG. The recombinant BCG-SIV_{mac} Gag was constructed and prepared as previously described (28).

Immunizations. Monkeys were inoculated with 10^8 CFU of recombinant BCG-SIV_{mac} Gag intradermally at four sites on the back in 0.4-ml total volumes. Each animal received two inoculations 19 weeks apart. Peptide immunization

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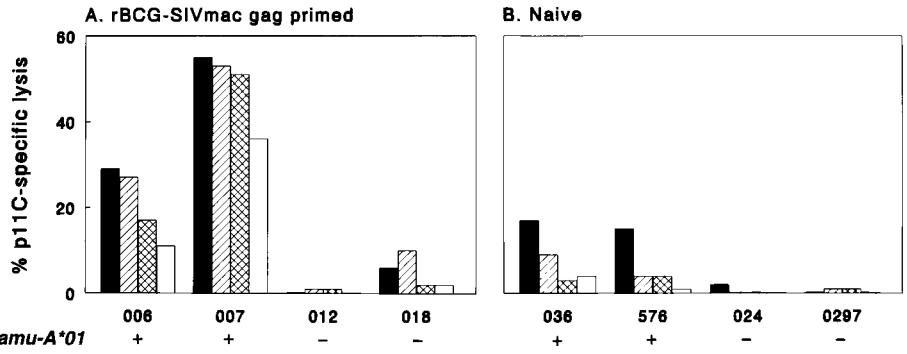

FIG. 1. p11C-specific PBL effector cell activity 8 weeks after p11C-liposome immunization. PBL (10^7) were cultured in 2 ml of medium containing 10 μg of SIV Gag p11C per ml. rIL-2 was added to the cultures on day 3 of culture; cells were assessed for lytic activity on day 7 of culture. E/T ratios were 20:1 (■), 10:1 (▨), 5:1 (▩), and 3:1 (□). The percentage of p11C-specific lysis was calculated as the percentage of lysis of p11C-pulsed target cells less the percentage of lysis of target cells pulsed with an irrelevant peptide. rBCG, recombinant BCG.

was done by intramuscular inoculation of 1 mg of p11C (EGCTPYDINQML) formulated with a lipid A-containing liposome with aluminum hydroxide as previously described (26). The first of these peptide inoculations was done 13 months following the last BCG-SIV_{mac} Gag inoculations.

Generation of CTL effector cells. Effector cells were Ficoll-diatrizoate density gradient centrifugation-isolated PBL. Aliquots of 10^7 PBL were placed in 12-well plates in 2 ml of RPMI 1640 medium containing 20% fetal calf serum (Flow Laboratories, McLean, Va.) and 10 μg of SIV_{mac} Gag p11C per ml. On day 3, 2 ml of medium containing recombinant interleukin-2 (rIL-2; provided by Hoffman-La Roche, Nutley, N.J.) at 40 U/ml was added to each culture. The cells were maintained in culture for 3 additional days. Dead cells were removed from these cultures by Ficoll-diatrizoate density gradient centrifugation immediately before assaying for cytotoxic function.

Isolation of $CD8^+$ and $CD4^+$ lymphocytes. $CD8^+$ and $CD4^+$ cells were isolated by positive selection with an anti-CD4 monoclonal antibody (MAb) (19Thy5D7, 1:500 dilution of ascites fluid; S. Schlossman, Dana-Farber Cancer Institute, Boston, Mass.) or an anti-CD8 MAb (7Pt3F9, 1:500 dilution of ascites fluid; S. Schlossman) followed by immunomagnetic bead separation (Dynal, Oslo, Norway). After 2 days in culture, the beads were removed from the purified $CD8^+$ or $CD4^+$ cultures by using a magnet. The viable cells were then isolated by Ficoll-diatrizoate density gradient centrifugation and assessed for effector function. The CD4- or CD8-depleted lymphocyte populations contained <2% $CD4^+$ or $CD8^+$ cells, respectively, as assessed by flow cytometry.

Cytotoxic assay. Target cells were either autologous or allogeneic *Mamu-A*01*$^+$ or *Mamu-A*01*$^-$ B-lymphoblastoid cell lines (B-LCL). These cells (10^6/ml) were incubated for 16 h at 37°C in a 5% CO_2 atmosphere with 0.5 μCi of Na_2CrO_4 (ICN, Irvine, Calif.) per ml and 50 μg/ml of either p11C or a control peptide, or a recombinant vaccinia virus expressing SIV_{mac} Gag (14) or recombinant vaccinia virus expressing the irrelevant equine herpesvirus 1 gH gene (13). Cells were then washed twice. These ^{51}Cr-labeled target cells were incubated for 5 h with effector cells at effector/target (E/T) ratios of 20:1, 10:1, 5:1, and 3:1. Spontaneous release varied from 10 to 20%. Specific release was calculated as [(experimental release − spontaneous release)]/(100% release − spontaneous release)] × 100.

Limiting-dilution assay. Relative frequencies of SIV-specific cytotoxic effector cells in PBL were determined by limiting-dilution analysis (19). Between 2.5×10^3 and 2.5×10^5 lymphocytes were assayed for cytotoxicity on ^{51}Cr-labeled B-LCL in a final volume of 0.2 ml. The B-LCL were infected with recombinant vaccinia-SIV_{mac} Gag or recombinant vaccinia virus expressing the irrelevant equine herpesvirus type 1 gH gene. Each concentration of effector cells was tested in 24 replicate wells. Supernatant fluids were collected and counted for radioactivity after a 5-h incubation at 37°C. A well was considered positive if ^{51}Cr release exceeded by 3 standard deviations the mean of control wells containing target cells alone. The effector cell frequencies were obtained by the statistical method of least-squares estimation (21). A single-hit Poisson distribution was used to model the generation of a positive response in the limiting-dilution assay. The 95% confidence limits were determined for each estimate. To simplify comparisons, effector cell frequencies presented in the text were normalized to number of effector cells per 10^4 lymphocytes.

SIV challenge. Monkeys were challenged intravenously with 2 ml of cell-free SIV_{mne} clone E11S at a dilution of 2×10^{-4} stock (provided by Raoul Benveniste, National Cancer Institute, Frederick, Md.). Previous titrations of this SIV stock in macaques indicated that this amount represented 20 animal infectious doses of virus (1, 5). Gag residues 182 to 190 (p11C) of SIV_{mac} (the virus used for construction of the recombinant BCG vector as well as the recombinant vaccinia virus-SIV Gag) and the challenge SIV_{mne} are identical (2).

Virus isolation. Concanavalin A-activated PBL were washed and depleted of CD8-expressing cells by incubation with the anti-CD8 MAb 7Pt3F9 followed by immunomagnetic bead separation. These $CD8^+$ lymphocyte-depleted PBL were cultured at 10^6 cells per ml for 2 weeks. Aliquots of culture supernatant were removed every 3 to 4 days. These aliquots were assessed for SIV p27 antigen as determined by enzyme-linked immunosorbent assay (Coulter p27 antigen assay).

Anti-SIV antibody assays. Anti-SIV_{mne} gp160 assays were performed by enzyme-linked immunosorbance with recombinant gp160 protein (kindly provided by S.-L. Hu, Seattle, Wash.). Custom SIV_{mac} Western immunoblot strips were purchased from Advanced Biotechnologies, Inc. (Columbia, Md.). Monkey sera were used at 0.020 ml per strip, which represented a 1:100 dilution.

Extraction of viral RNA and sequence analysis. Limited sequence analysis was performed on SIV isolated from animals 006, 012, 018, and 008. These viruses were isolated from PBL obtained 4 weeks postchallenge. The first third of the p28 region of *gag* was amplified from viral RNA, extracted as previously described (*gag* RNA extraction), using the Gene Amp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) and the CloneAmp System (Life Technologies, Inc., Gaithersburg, Md.). The oligonucleotide primer used in the reverse transcriptase reaction was 5′-$(CAU)_4$CCAATCTGCAGCCTCCTCGT-3′. The 13th nucleotide represented the SIV_{mne} *gag* gene nucleotide pair 642. The second primer used in the PCR mix addition was 5′-$(CUA)_4$ACTATACCCACCTACCATTA AGC-3′, in which the 13th nucleotide represented nucleotide pair 428 in the SIV_{mne} *gag* gene (11). PCR products were cloned by reacting them with uracil DNA glycosylase while annealing to the pAMP vector plasmid. Sequence analysis of verified insert clones was performed as previously described, using a modification of the Sequenase version 2.0 (U.S. Biochemical) protocol (4).

RESULTS

Greater SIV Gag-specific effector T-cell responses were elicited through combined modality immunization than through peptide immunization alone. We first determined whether secondary SIV-specific effector T-cell responses could be elicited through immunization. To this end, we assessed a combined modality approach to the vaccine induction of SIV-specific

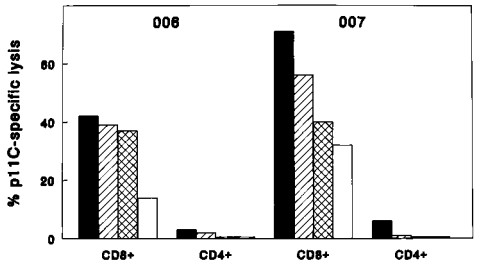

FIG. 2. p11C-specific effector cells generated by combined modality immunization are $CD8^+$. Effector cells were expanded in medium containing rIL-2 and 10 μg of p11C per ml and then fractionated into $CD4^+$ or $CD8^+$ effector cells by positive selection using MAbs and immunomagnetic beads. E/T ratios were 20:1 (■) 10:1 (▨), 5:1 (▩), and 3:1 (□). The percentage of p11C-specific lysis was calculated as the percentage of lysis of p11C-pulsed target cells less the percentage of lysis of target cells pulsed with an irrelevant peptide.

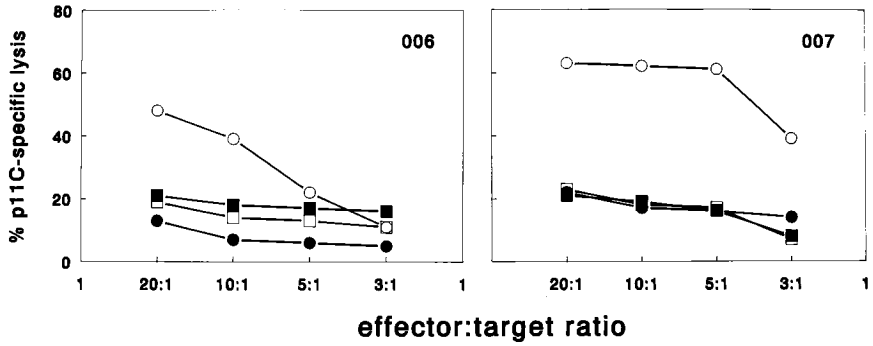

FIG. 3. p11C-specific effector cells generated by combined modality immunization are *Mamu-A*01* restricted. Target cells were allogenic B-LCL, either *Mamu-A*01*$^+$ (circles) or *Mamu-A*01*$^-$ (squares), pulsed with p11C (open symbols) or an irrelevant control peptide (closed symbols). The percentage of p11C-specific lysis was calculated as the percentage of lysis of p11C-pulsed target cells less the percentage of lysis of target cells pulsed with an irrelevant peptide.

CTL: live recombinant BCG priming, followed by peptide-liposome boosting. Two groups of rhesus monkeys were used in this study; one group had four normal monkeys that were immunized twice with a recombinant BCG-SIV Gag, and the second group had four naive monkeys. There were two *Mamu-A*01*$^+$ monkeys in each experimental group. All monkeys received a single intramuscular inoculation of 1 mg of p11C formulated with a lipid A-containing liposome with aluminum hydroxide 13 months following BCG-SIV Gag inoculation.

p11C-specific lytic activity was assessed in PBL of these monkeys following in vitro p11C stimulation. Prior to the p11C-liposome boosting, PBL of the two *Mamu-A*01*$^+$ monkeys immunized with BCG-SIV Gag demonstrated persistent, low-level p11C-specific lytic responses, 12 and 14% specific lysis at an E/T ratio of 20:1. PBL of the *Mamu-A*01*$^-$ monkeys previously immunized with BCG-SIV Gag and those of the unprimed monkeys did not mediate p11C-specific lysis. By 8 weeks after p11C-liposome immunization, PBL from all the *Mamu-A*01*$^+$ monkeys, both those previously immunized with recombinant BCG-SIV Gag and the previously unprimed animals, demonstrated a p11C-specific effector response; PBL of the similarly immunized *Mamu-A*01*$^-$ monkeys in both experimental groups did not mediate p11C-specific lysis (Fig. 1). PBL obtained from the previously immunized *Mamu-A*01*$^+$ monkeys showed greater p11C-specific lytic activity following peptide-liposome boosting than did PBL of the previously unprimed *Mamu-A*01*$^+$ monkeys (Fig. 1).

Effector cells elicited by combined modality immunization are CTL. The p11C-specific effector cells generated through combined modality immunization were further characterized. MAbs and immunomagnetic beads were used to separate CD4$^+$ and CD8$^+$ cells from PBL of the combined modality immunized *Mamu-A*01*$^+$ monkeys. CD8$^+$ lymphocytes mediated p11C-specific lysis, while CD4$^+$ lymphocytes did not exhibit this effector cell function (Fig. 2). The p11C-specific effector cells from the PBL of the p11C-liposome-immunized *Mamu-A*01*$^+$ monkeys previously primed with BCG-SIV Gag lysed p11C-pulsed, *Mamu-A*01*$^+$ allogeneic target cells but did not lyse p11C-pulsed, *Mamu-A*01*$^-$ target cells (Fig. 3). Therefore, combined modality immunization of *Mamu-A*01*$^+$ rhesus monkeys elicited CD8$^+$, MHC class I-restricted CTL.

Although these studies indicated that this mode of immunization elicited p11C-specific CTL, it was important to determine whether these effector cells also lysed virus-infected target cells. The capacity of combined modality immunization to elicit effector cells that recognize virus-infected cells was assessed by using target cells infected with a recombinant vaccinia virus encoding the SIV *gag* gene. PBL from the *Mamu-A*01*$^+$ monkeys that were immunized by a combined modality approach mediated SIV Gag-specific lysis (Fig. 4). Such SIV Gag-specific lysis was not demonstrated by PBL of the similarly immunized *Mamu-A*01*$^-$ monkeys. Thus, monkeys immunized by this combined modality approach generated CTL capable of recognizing endogenously processed viral protein.

Combined modality immunization elicited a high frequency of SIV-specific CTL. Limiting-dilution effector precursor frequency assays were performed from PBL of the experimental animals, using target cells infected with vaccinia virus-SIV Gag (Table 1). SIV Gag-specific CTL precursors in PBL of all recombinant BCG-SIV Gag-primed animals were detected before boosting; CTL precursors were not detected in PBL of the naive animals at that time. After boosting, SIV Gag-specific CTL precursors were increased in frequency in the *Mamu-A*01*$^+$ monkeys. CTL precursor frequencies in PBL of the *Mamu-A*01*$^-$ monkeys were unchanged, and these effector frequencies were not detected in PBL of the *Mamu-A*01*$^-$ naive monkeys. The number of experimental monkeys in each group was small, and there was, as expected in these outbred animals, variability in SIV Gag-specific effector frequency between similarly immunized animals. Nevertheless, it appears as though the CTL precursor frequencies in PBL of the *Mamu-A*01*$^+$ monkeys immunized by this combined modality approach appeared to be higher than those of the *Mamu-A*01*$^+$ monkeys immunized with peptide-liposome alone. Moreover,

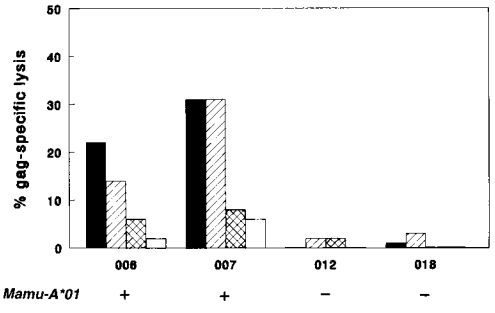

FIG. 4. Effector cells generated by combined modality immunization recognize endogenously processed SIV Gag protein. Target cells were prepared by infecting B-LCL with vaccinia virus recombinants. The recombinant SIV Gag-vaccinia virus construct contained the entire Gag protease-encoding region from the 251 isolate of SIV_{mac}. The control vaccinia virus encoded the equine herpesvirus type 1 gH gene. The percentage of SIV Gag-specific lysis was calculated as the percentage of lysis of recombinant-vaccinia SIV Gag-infected target cells less the percentage of lysis of the control vaccinia virus-infected target cells. E/T ratios were 20:1 (■), 10:1 (▨), 5:1 (▩), and 3:1 (□).

TABLE 1. SIV Gag-specific CTL precursor frequency in PBL of immunized and SIV-infected rhesus monkeys

Immunization or infection group	Monkey	*Mamu-A*01* status	SIV Gag-specific effector cells/10^4 PBL[a]	
			Before p11C boosting	After p11C boosting
Recombinant BCG-SIV Gag immunization	006	+	0.016 (0.013–0.020)	0.117 (0.073–0.160)
	007	+	0.013 (0.009–0.017)	0.063 (0.022–0.100)
	012	−	0.010 (0.007–0.018)	0.011 (0.008–0.013)
	018	−	0.074 (0.060–0.088)	0.080 (0.065–0.090)
Naive	036	+	0.000 (0.000–0.000)	0.035 (0.015–0.086)
	576	+	0.000 (0.000–0.000)	0.007 (0.003–0.016)
	024	−	0.000 (0.000–0.000)	0.000 (0.000–0.000)
	0297	−	0.000 (0.000–0.000)	0.000 (0.000–0.000)
SIV_{mac} infection	008	+	0.150 (0.101–0.185)	
	179	+	0.010 (0.002–0.011)	
	467	+	0.036 (0.030–0.042)	

[a] Limiting-dilution CTL assays were performed with PBL from vaccinated and SIV_{mac}-infected monkeys. Ninety-five percent confidence limits are shown in parentheses.

the SIV Gag-specific CTL precursor frequencies in PBL of the combined modality immunized *Mamu-A*01*$^+$ monkeys were within the same range as those of SIV-infected monkeys.

SIV challenge of monkeys with a high-frequency single-epitope SIV Gag-specific CTL response. Western blot analysis detected weak reactivity to SIV p27 and the Gag precursor in the serum of only one of the vaccinated monkeys, monkey 012 (Fig. 5). Therefore, this cohort of immunized animals had, for all practical purposes, only an SIV-specific CTL response. This situation provided the opportunity to determine whether a single SIV epitope-specific CTL response alone could confer protection against live SIV challenge. Six rhesus monkeys were used in an SIV challenge experiment: the two *Mamu-A*01*$^+$ rhesus monkeys immunized first with recombinant BCG-SIV Gag and then p11C-liposome (monkeys 006 and 007); the two similarly immunized *Mamu-A*01*$^-$ animals (monkeys 012 and 018); and two naive control monkeys, one *Mamu-A*01*$^+$ (637) and the other *Mamu-A*01*$^-$ (008).

Twelve months following their first p11C-liposome immunization, the BCG-SIV Gag–p11C-immunized rhesus monkeys were boosted again with p11C formulated with a lipid A-containing liposome in aluminum hydroxide. As observed previously, the two *Mamu-A*01*$^+$ but not the *Mamu-A*01*$^-$ monkeys developed a strong p11C-specific effector cell response, with p11C-specific lysis of 37 and 58% measured at an E/T ratio of 20:1 in monkeys 006 and 007, respectively. Two weeks following an additional p11C-liposome immunization of these same monkeys, 20 animal infectious doses of the E11S isolate of SIV_{mne} were injected intravenously as a cell-free challenge of the four immunized and two naive control monkeys.

PBL of the three *Mamu-A*01*$^+$ rhesus monkeys were assessed on days 2, 8, 15, and every 2 weeks thereafter until day 56 following virus challenge for the persistence of a p11C-specific CTL response (Table 2). The immunized *Mamu-A*01*$^+$ monkey 006, which had the lower frequency SIV Gag-specific effector response at the time of challenge, demonstrated a gradual rise in p11C-specific CTL beginning approximately 2 weeks following virus challenge. The immunized *Mamu-A*01*$^+$ monkey 007, which had the higher SIV Gag-specific effector response at the time of challenge, showed a gradual fall in this CTL response until day 28 following virus

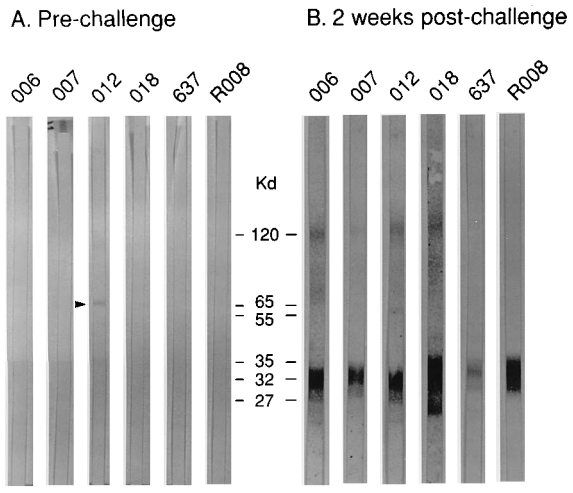

FIG. 5. Detection of seroconversion to SIV antigen reactivity in rhesus monkey sera by Western immunoblot analysis. Numbers in the center indicate molecular masses of Western blot-reactive SIV proteins that are detected. (A) Reactivity of sera from the six animals prechallenge. The arrowhead to the left of the lane for animal 012 indicates reactivity that was observed postimmunization and before challenge. (B) Reactivity of sera from the six animals at 2 weeks postchallenge. The major reactivity of these positive sera was with the *gag* products, and this seroconversion reactivity is similar to that previously observed and described for SIV-infected rhesus monkeys (21a). The numbers at the top identify the monkeys. Sera were tested through 15 weeks postchallenge. Nonvaccinated monkey number 637 was the only one whose sera declined in reactivity with time.

TABLE 2. p11C-specific CTL in PBL of *Mamu-A*01*$^+$ monkeys following SIV challenge[a]

Immunization group	Monkey	E/T	p11C-specific lysis at given days postchallenge						
			0	2	8	15	28	42	56
Recombinant BCG-SIV Gag–p11C	006	20	38	29	41	47	63	65	71
		10	28	20	33	39	60	59	56
		5	10	11	25	30	47	44	50
		3	0	2	14	16	36	39	30
Recombinant BCG-SIV Gag–p11C	007	20	64	76	65	26	15	27	33
		10	49	61	47	14	9	18	21
		5	21	45	30	6	9	11	14
		3	16	10	18	2	4	7	4
Naive	637	20	0	0	0	0	8	18	16
		10	0	0	0	0	2	11	9
		5	0	0	0	0	0	6	4
		3	0	0	0	0	0	2	1

[a] Effector cells were stimulated in vitro with 10 μg of SIV Gag p11C per ml and expanded in medium containing rIL-2.

TABLE 3. SIV Gag-specific CTL frequency in PBL of monkeys following SIV challenge

Immunization group	Monkey	*Mamu-A*01*	SIV Gag-specific effector cells/10^4 lymphocytes at given wk postchallenge[a]				
			0	1	2	4	8
Recombinant BCG-SIV Gag–p11C	006	+	0.041 (0.035–0.047)	0.060 (0.053–0.068)	0.101 (0.088–0.114)	0.166 (0.155–0.177)	0.177 (0.155–0.199)
	007	+	0.146 (0.124–0.168)	0.189 (0.165–0.213)	0.116 (0.104–0.128)	0.040 (0.029–0.051)	0.061 (0.047–0.075)
Recombinant BCG-SIV Gag–p11C	012	−	0.011 (0.006–0.016)	0.020 (0.014–0.026)	0.046 (0.034–0.058)	0.039 (0.027–0.051)	0.048 (0.034–0.062)
	018	−	0.022 (0.019–0.025)	0.044 (0.030–0.058)	0.066 (0.056–0.075)	ND[b]	0.089 (0.077–0.101)
Naive	637	+	—[c]	—	0.009 (0.004–0.014)	0.010 (0.004–0.016)	0.022 (0.010–0.034)
	R008	−	—	—	0.014 (0.003–0.025)	0.022 (0.011–0.033)	0.021 (0.014–0.028)

[a] Ninety-five percent confidence limits shown in parentheses. Autologous target cells were used in all assays.
[b] ND, not done.
[c] —, Negative.

challenge. Thereafter, this animal began demonstrating a rise in its SIV Gag-specific effector response. The naive control *Mamu-A*01*$^+$ monkey 637 first had a detectable p11C-specific CTL response in its PBL 28 days following infection. This response increased in magnitude in the following weeks.

The PBL from all six animals were assessed for SIV Gag-specific precursor CTL frequency every 2 weeks following virus challenge (Table 3). This response in the two *Mamu-A*01*$^+$ vaccinated animals paralleled the evolution of the bulk p11C-specific CTL response in PBL. In the two *Mamu-A*01*$^-$ vaccinated monkeys, a gradual rise in the response was noted during the first 8 weeks following virus challenge. In the two naive monkeys, a low-frequency SIV Gag-specific response was first noted 2 weeks following challenge; this response rose in both monkeys over the ensuing weeks.

As suggested by these rising SIV-specific CTL responses, all six animals were infected following virus challenge. The monkeys developed anti-SIV gp160-specific antibody responses (data not shown). Two weeks postchallenge, plasma from all animals was Western blot reactive with SIV Gag (Fig. 5). All plasma samples remained Western blot positive through 15 weeks postchallenge except those of control monkey 637, which declined in reactivity with SIV Gag over time.

Virus was isolated from all the animals. During the first 56 days following virus challenge, SIV_{mne} was isolated once from monkeys 007 and 637, twice from monkeys 012 and 008, and six times from monkey 018. The variability in virus isolations from these monkeys was likely attributable to the difficulty always seen in isolating the E11S clone of SIV_{mne} from any experimentally infected macaques. To determine whether the virus isolated from these monkeys might have mutated to escape recognition by the vaccine-elicited, p11C-specific CTL, the virus isolated 4 weeks following inoculation from three of these animals (monkeys 006, 018, and 008) was characterized. Eight to ten viral *gag* clones were sequenced from each virus from each of the three monkeys. All clones encoded a p11C sequence identical to that of the infecting virus and the immunizing sequence. These data, therefore, indicate that a vaccine-elicited, single-epitope-specific CTL response does not provide protection against an intravenous, cell-free SIV challenge.

DISCUSSION

Previous studies in the SIV-macaque model demonstrated that SIV Gag-specific CTL can be elicited in rhesus monkeys by immunization with live vector vaccines including recombinant vaccinia virus and recombinant BCG (20, 28). In the present experiments, the detection of SIV Gag-specific CTL persisting in monkeys greater than a year following immunization with recombinant BCG-SIV Gag alone indicates that long-lived virus-specific immunity can be elicited by this vector system. Other previous experiments in the SIV-macaque model have shown that SIV Gag-specific CTL can also be elicited by immunization with peptide, formulated in a proteoliposome or simply in incomplete Freund's adjuvant (12, 29). In the present studies, we demonstrate that primary SIV Gag-specific CTL can also be elicited by immunization with peptide formulated in a lipid A-containing liposome in aluminum hydroxide. Thus, a variety of formulations of this SIV Gag peptide can facilitate its transport into the MHC class I presentation pathway.

In prior experiments, we had shown that SIV Gag-specific CTL can be elicited in rhesus monkeys by priming with recombinant vaccinia virus-SIV Gag and boosting with noninfectious virus-like particles (19). However, in that study we did not assess CTL induction by immunization with virus-like particles alone, nor did we compare the relative efficacy of single and combined modality approaches to vaccination. In the present experiments, we demonstrate, in both bulk CTL assays and limiting-dilution effector frequency assays, that a combined modality approach to CTL induction may elicit a more potent CTL response that can be generated following vaccination with peptide alone. It should be noted, however, that multiple immunizations with one of these vaccine modalities may have the capacity to at least transiently increase a CTL response to this same degree. In fact, we previously showed that while a single inoculation of BCG-SIV Gag elicits a SIV Gag-specific CTL response that can be detected only after in vitro peptide stimulation of PBL, this response could be transiently detected directly in PBL ex vivo following a second BCG-SIV Gag inoculation without such a peptide restimulation (28). Our ability to expand a precursor pool of SIV Gag-specific CTL through combined modality vaccine approaches is lent further support by the rapid expansion of these CTL responses in the vaccinated monkeys following SIV infection (Tables 2 and 3).

It is interesting that no evidence was seen in this study for selective pressure exerted on the virus by the preexisting CTL response. It has been suggested that HIV-1 may escape recognition by CTL through mutation. This phenomenon has been reported for an HLA B8-restricted Gag epitope in a limited number of HIV-infected patients (17). Moreover, the infusion of large numbers of autologous cloned HIV Nef-specific CTL in one HIV-infected subject coincided with an increase in viral burden and an emergent population of virus with a mutation in *nef* that allowed the virus to escape from recognition by the infused CTL (7). We have previously demonstrated that the SIV Gag-specific CTL response in rhesus monkeys that share the MHC class I allele *Mamu-A*01* is remarkably restricted. Gag recognition by CTL in these animals occurs only in the context of *Mamu-A*01* and is limited to a single epitope, amino acid residues 182 to 190 of the SIV Gag protein (14).

These features should maximize the CTL-mediated selective pressure driving mutations in an epitope of the virus. The fact that mutations at that epitope of SIV did not occur in these vaccinated *Mamu-A*01*$^+$ monkeys, however, is consistent with our inability to demonstrate the emergence of such SIV variants in chronically SIV-infected *Mamu-A*01*$^+$ monkeys (3). The region of Gag defined by p11C may be essential for the integrity of the virus and structurally constrained such that mutations in this domain may not be consistent with replication competency of the virus.

While disappointing, our inability to elicit protective immunity in this study should probably not be surprising. Although CTL are likely to play a central role in containing the spread of cell-associated SIV, it may have been naive to assume that a CTL response with only a single epitope specificity might provide protective immunity against a live virus challenge. Furthermore, CTL recognize endogenously processed viral protein bound to MHC class I molecules on the surface of infected cells (22). Therefore, such a cellular response might not be expected to contain cell-free virus. Neutralizing antibody is probably needed to achieve sterilizing immunity against cell-free intravenous virus challenge. Therefore, more optimal immunity might be expected from a vaccine that elicits potent neutralizing antibody of broad specificity and CTL with specificities for multiple viral epitopes.

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