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Type 1 CD4$^+$ T-Cell Help Is Required for Induction of Antipeptide Multispecific Cytotoxic T Lymphocytes by a Lipopeptidic Vaccine in Rhesus Macaques

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We have optimized the induction of antiviral cytotoxic T lymphocytes (CTL) in rhesus macaques by a lipopeptide vaccine containing seven peptides from simian immunodeficiency virus (SIV) Nef and Gag proteins and a strong T-helper peptide from tetanus toxoid (TT) that is promiscuous in humans (peptide TT 830-846). Two of the eight immunized macaques showed T-helper (Th) cell proliferation and a specific synthesis of gamma interferon in response to TT 830-846 peptide. They also showed multispecific cytotoxic activity against three to five of the immunizing SIV peptides. These results show the importance of a strong specific type 1 Th response for inducing a multispecific CTL response in vivo, which is essential for the development of an anti-human immunodeficiency virus vaccine.

Virus-specific CD8$^+$ responses are essential for immune protection against several viruses (8, 34, 37). Hence, successful vaccines must induce cellular immunity mediated by CD8$^+$. Cytotoxic T lymphocytes (CTLs) are involved in the control of the viral load during human immunodeficiency virus (HIV) infection (4, 7, 17, 18) and seem to be very important in vaccine-induced protection (12). However, as they exert considerable selective pressure in both primary and late-stage HIV infections, they may select for escape mutant viruses (5, 14, 28). We have demonstrated that the induction by lipopeptides of CD8$^+$ CTLs recognizing only one epitope in the simian immunodeficiency virus (SIV)-infected macaque model is not sufficient to protect against SIV and may favor the selection of variant viruses and the emergence of escape mutant viruses (23). Thus, the induction of multispecific CTLs that recognize several virus isolates by giving an appropriate vaccine is likely to be essential to prevent selection of mutant or variant viruses.

We have now immunized eight rhesus macaques (*Macaca mulatta*) (92102, 92105, 92109, 92117, 92120, 92125, 92127, and 92129) with tetanus toxoid (TT) (500 μg per monkey) in incomplete Freund adjuvant (IFA) (three subcutaneous and intramuscular injections given at 1-month intervals). Indeed, initial vaccination with a carrier protein induces helper T (Th) memory cells that may then be exploited by using selected relevant T epitopes from the same protein to boost B cell and CTL responses (15, 31). The proliferation of peripheral blood mononuclear cells (PBMCs) against TT was measured by monitoring ^{3}H-labeled thymidine incorporation 1 month after the last immunization; it was significant for all macaques (data not shown). Five months later, there was a significant Th-specific response to lipopeptide TT 830-846 in only two macaques, 92109 and 92129 (data not shown).

Seven months after the last TT immunization, the macaques were given three subcutaneous injections of a mixed-micelle formulation of eight lipopeptides in sterile water (500 μg of each lipopeptide) without any adjuvant at 1-month intervals. This immunization procedure was thus compatible with human vaccination. Five sequences of lipopeptides were selected from the SIV Nef (LP1, amino acids [aa] 101 to 126; LP2, aa 125 to 147; LP3, aa 155 to 178; LP4, aa 201 to 225; and LP5, aa 221 to 247), and two were selected from the Gag protein (LP6, aa 165 to 195, and LP7, aa 246 to 281). These were identical to sequences previously reported (6, 7, 10, 23) except for the introduction of an additional N^{ε}-palmitoyl-lysylamide residue at the C terminus (23). In addition, to improve CTL induction, a lipopeptide containing promiscuous human Th epitope from TT-derived peptide, aa 830 to 846, Ac-QYIKANSKFIGITELKK, referred to herein as LP-TT; this was preferred to peptide 830-843 because of its greater solubility and was synthesized with a modification of the N-terminal extremity by an acetyl group, to avoid any heterogeneity that might be produced by formation of the pyroglutamyl analog upon storage. The mixed-micelle formulation was obtained by dissociating each component in concentrated acetic acid (80%) before mixing and sterilization by filtration. Dilution resulted in the formation of mixed micelles or aggregates that, statistically, contained each of the constituents.

Lipopeptide TT 830-846 Th-specific responses were assessed 6 months after the last lipopeptide immunization; they showed that the same two macaques (92109 and 92129) always had strong responses to lipopeptide TT 830-846 (data not shown). We therefore investigated whether these results depended upon a particular type 1 or type 2 profile and analyzed the CD4$^+$ or CD8$^+$ nature of these T cells. We first assessed lymphokine production by PBMCs which had undergone a short stimulation in vitro with LP-TT by using a sensitive enzyme-linked immunospot (ELISPOT) assay for gamma interferon (IFN-γ) adapted from Scheibenbogen et al. (33). Only the PBMCs from the two macaques showing proliferative assay response (92109 and 92129) specifically synthesized IFN-γ (Fig. 1a). We then confirmed the CD4$^+$ nature of the effector T cells that

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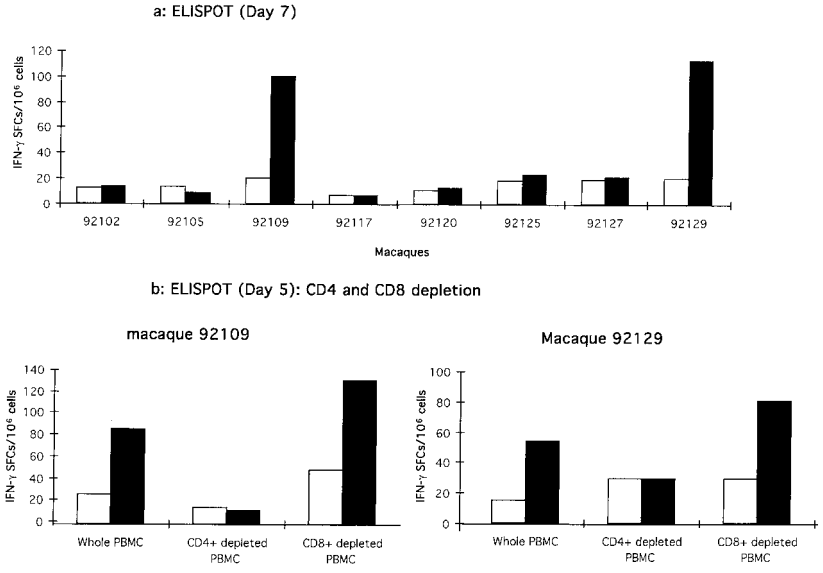

FIG. 1. Synthesis of IFN-γ by $CD4^+$ lymphocytes after LP-TT stimulation in macaques 92109 and 92129. (a) Helper peptide-specific IFN-γ SFCs in PBMCs from the eight macaques were analyzed after the third mixed-micelle lipopeptide immunization. The ELISPOT assay was performed 7 days after one short in vitro stimulation. Briefly, PBMCs (2.5×10^6/ml) were cultured for 3 days in 24-well microtiter plates (Costar, Cambridge, Mass.) in complete medium, with 5 μM LP-TT or with 5 μM irrelevant LP (Nef HIV 66-97) (LP-i). IL-2 (Boehringer, Mannheim, Germany) was then added to each well (10 IU/ml), and incubation was continued for 4 days. Effector cells were then washed, counted, and seeded in duplicate in 96-well nitrocellulose plates (Multi-Screen HA; Millipore, Bedford, Mass.) that had been coated with the mouse anti-human IFN-γ capture monoclonal antibody (Genzyme, Russelheim, Germany) (8 μg/ml in carbonate buffer) and blocked with complete medium. Effector cells were incubated at 10^5 and 2×10^5 per well with 5 μM LP-i (white columns) or 5 μM LP-TT (black columns) for 48 h in complete medium containing 20 IU of IL-2/ml. The ELISPOT assay was performed as previously described (33). Responses were considered significant if there were a minimum of five SFCs per well and if this number was at least twice that obtained with the negative control. (b) A depletion assay was done using anti-CD4 or anti-CD8 monoclonal antibodies (MAbs) on the Th cell responder macaques (90109 and 92129) to obtain $CD4^+$-depleted PBMCs and $CD8^+$-depleted PBMCs, together with whole PBMCs. PBMCs were incubated for 30 min with cocktails of anti-human CD8 (DAKO, Glostrup, Denmark; Becton Dickinson, Mountain View, Calif.; and Ortho Diagnostic Systems, Raritan, N.J.) (1 μl of each/10^6 cells) or cocktails of human anti-CD4 (DAKO; Sigma Chemical Co., St. Louis, Mo.; and Ortho Diagnostic Systems) (1 μl of each/10^6 cells) MAbs coated on Dynabeads in 500 μl of complete medium on ice (BioMag goat anti-mouse immunoglobulin G; PerSeptive Biosystems, Framingham, Mass.). Conjugate-coated cells were then removed with a magnet (Dynal) and cultured in vitro for 5 days. Finally, they were tested in the ELISPOT assay.

secreted IFN-γ in response to lipopeptide TT 830-846 by using the ELISPOT assay to measure IFN-γ levels in PBMCs, $CD4^+$-depleted PBMCs, and $CD8^+$-depleted PBMCs (Fig. 1b). The LP-TT-specific response of the $CD8^+$-enriched population was completely abrogated. Conversely, depletion of $CD8^+$ cells did not decrease the number of IFN-γ spot-forming cells (SFCs), indicating that the IFN-γ secretion induced by LP-TT was mediated by $CD4^+$ T cells.

The lipopeptide-induced CTL responses were examined after the last mixed-micelle immunization by stimulating macaque PBMCs with a mixture of the seven long free peptides without the helper TT 830-846 epitope and testing them against autologous B lymphoblastoid cell lines (B-LCLs) sensitized by the same long peptides. On the day of the chromium release test (CRT), effector cells were >70% $CD8^+$ T cells for every test performed, as might be expected for class I-restricted antigen-specific CTLs. Most (seven) of the eight immunized macaques had CTL activity (Fig. 2), and macaques 92109 and 92129 had strong and multispecific CTL responses to five and three long peptides, respectively. We tested overlapping short peptides (8 to 11 aa) spanning the sequence of the long peptides (Table 1) to identify six epitopes recognized by CTLs from macaque 92109, three of which (NEF 169-178, NEF 215-225, and GAG 266-275) were very strongly recognized. Three others caused less lysis. Similarly, the CTLs from macaque 92129 recognized four peptides (NEF 128-136, NEF 201-211, NEF 211-219, and NEF 169-178). Macaque 92127 had CTLs that recognized two long peptides with a cytotoxic activity lower than those of peptides from macaques 92109 and 92129 (Fig. 2). Macaques 92125, 92120, 92102 and 92105 had CTLs that recognized a single peptide, while the CTLs from macaque 92117 recognized no peptide. A maximum of one short peptide in every long peptide recognized was identified for macaques 92125, 92127, and 92105 (Table 1). In contrast, in macaques 92102 and 92120, no short epitopic peptides within long peptides were identified. CTL activities persisted in all the macaques for 9 to 10 months after the last lipopeptide immunization and did not result from in vitro induction of primary CTL responses, since they were not detected after antigen-specific stimulation of naive PBMCs from the seven CTL responders (data not shown). The multispecific activity in the two responder macaques was then associated with significant type 1 Th (Th1) responses to the TT 830-846 peptide after immunization with either TT or lipopeptide ($P < 0.05$ by the chi-square test). A recent in vivo study has shown that T helper subset cells with Th1 profiles regulate both the sensitivity and the frequency of epitope-specific CTL responses in mice (29). A relationship between significant Th responses and strong, persistent CTLs with high frequencies of CTL precursors (CTLp) in vivo in mice (3, 16, 19, 24, 32, 35) and humans vaccinated with lipopeptides against chronic hepatitis B virus infection (20, 39) has also been described.

Synthetic peptides modified at one end by addition of a lipid moiety are highly immunogenic for T and B cell responses in vivo (2, 11, 22). Lipopeptides also facilitate the presentation of peptides by major histocompatibility complex (MHC) class I molecules (11), which may be due to their ability to rapidly cross the cell membrane and enter the cytoplasm of intact cells (21, 38). Last, large synthetic lipopeptides can be processed in a manner similar to whole exogenous proteins and become

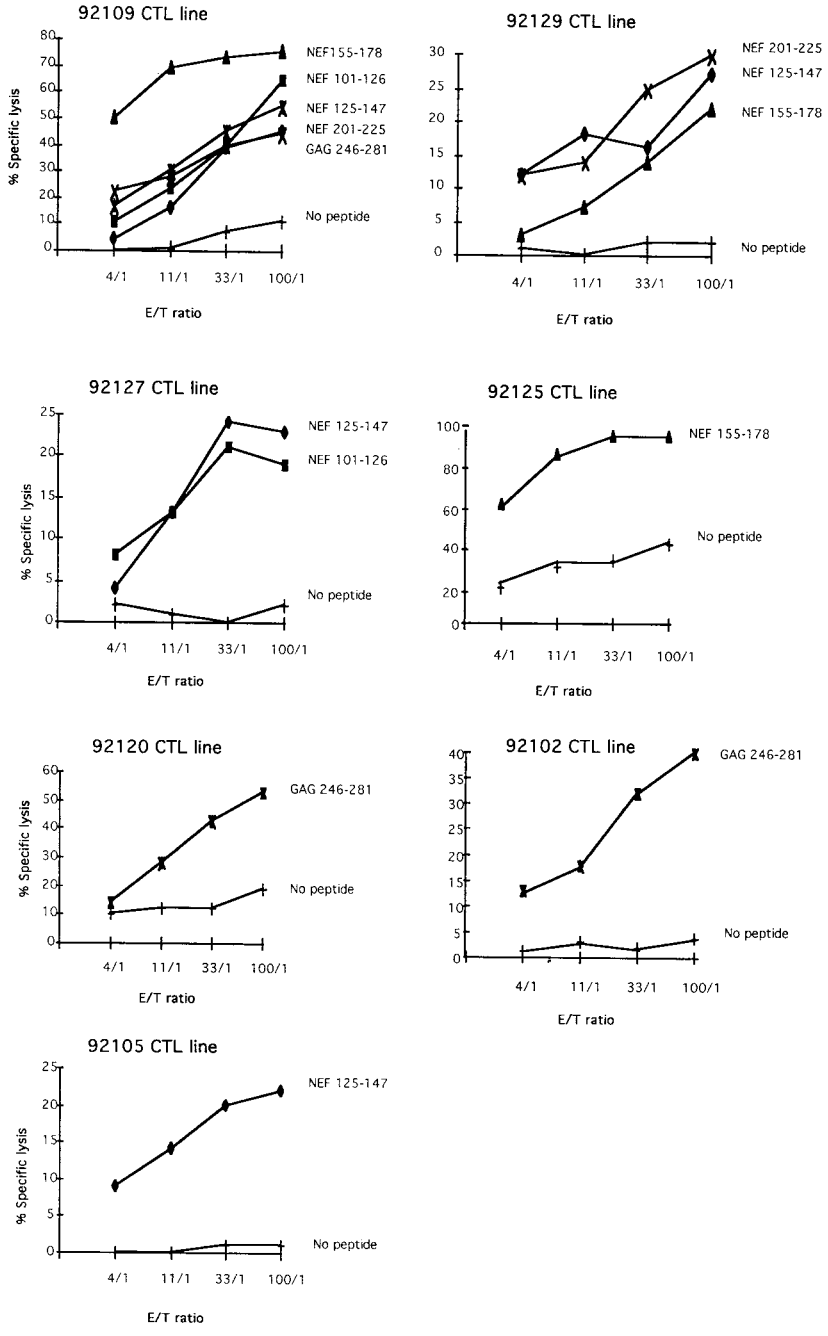

FIG. 2. Cytotoxic activities of the seven responder macaques. Anti-peptide CTL lines were obtained by pulsing for 2 h macaque PBMCs (10×10^6 cells/ml) with a mixture of the five Nef and two Gag free long peptides (10 μM each) corresponding to the immunizing lipopeptides. The cells were then washed and resuspended (10^6/ml) in complete medium and incubated in 24-well microtiter plates. After 3 days of incubation, IL-2 was added to each well (10 IU/ml). On days 7 and 14, effector cells were washed and diluted to 10^6/ml, placed in new plates, and stimulated with irradiated peptide-pulsed (10 μM each) autologous PBMCs (10×10^6/ml) for 2 h and then diluted to 10^6/ml in complete medium containing 20 IU of IL-2/ml (effector/stimulator ratio, 1:1). A CRT was performed after the third stimulation of CTL lines. The target cells were autologous B-LCLs (immortalized by the herpesvirus papio) alone or incubated overnight with various long peptides (10 μM/ml). The CRT was considered positive if the specific ^{51}Cr release observed in the presence of peptide-pulsed target cells exceeded by 10% that observed for B-LCLs without peptide at two effector/target (E/T) ratios. Only the positive cytotoxic responses against peptide-sensitized target cells of the seven responder macaques are shown.

associated with MHC class II molecules. We chose to use the promiscuous helper peptide TT 830-843 that binds to several HLA-DR molecules (20, 26, 27, 39) in this study. Other promiscuous peptides could be used in humans, including PADRE epitopes, which bind to 14 different HLA-DR molecules (9). This degenerate binding specificity of peptides could overcome the problem of the extreme polymorphism of HLA-DR molecules in humans. We used the promiscuous TT peptide because it provides adequate T cell help to induce CTLs, as shown in a human vaccine trial against chronic hepatitis B virus (20, 39). We believe that an immunizing formulation with mixed micelles that statistically contain each of the constituents allows the physical association of Th and CTL peptides and may favor the presentation of peptides to T helper lym-

TABLE 1. Epitopic specificities found in five immunized macaques

Effector cells[a] from macaque	Target cells[b]	% Specific lysis[c] at the E/T ratio[d] of:			
		100:1	33:1	11:1	4:1
92109	None	25	21		
	Nef 169-178	89[e]	75		
	None	14	11		5
	Nef 215-225	41	36		22
	None	19	9	7	3
	Gag 266-275	40	24	16	6
	None	14	11	11	5
	Nef 101-110		26	22	16
	Nef 128-136	28	23		
	None	41	34	41	27
	Nef 116-126	57	45	48	34
92129	None	8	3	2	0
	Nef 128-136	46	34	28	9
	Nef 201-211	22	16	16	5
	Nef 211-219	19	16	10	4
	None	52	44	30	
	Nef 169-178	65	54	33	
92125	None	38	34	25	19
	Nef 169-178	86	87	70	54
92127	None		28	21	
	Nef 116-126		43	32	
92105	No peptide	22	14	12	2
	Nef 128-136	34	24	13	5

[a] CTL cell lines were obtained from PBMCs of the five immunized macaques following specific stimulation with the seven long peptides in vitro.

[b] Target cells were autologous B-LCLs immortalized by the herpesvirus papio and incubated with short peptides (10 μM).

[c] Target cells (5×10^3) were labeled with ^{51}Cr and incubated for 4 h with various numbers of target cells.

[d] E/T ratio, effector-to-target ratio.

[e] CRT was considered positive if the specific ^{51}Cr release observed in the presence of peptide-pulsed target cells exceeded by 10% that observed on B-LCLs without peptide at two E/T ratios.

phocytes and CTLs by the same antigen-presenting cell, which is essential for optimal productive interactions and collaboration between Th cells and CTLs (36).

The breadth of the CTL peptide recognition spectrum in only two macaques (92109 and 92129) in this study could have been due to the heterogeneity of their MHC class I molecules. This is unlikely in the light of the results of much larger studies including our previous experiments (7, 23). We obtained 16 CTL responders from 22 macaques immunized with lipopeptides and found that 14 of them had mono- or bispecific CTL responses. In addition, these two macaques belong to the same cohort as the six Th nonresponders, and all these macaques may share the same MHC class I molecules. Therefore, induction of multispecific CTLs is unlikely to be due to particular MHC class I molecules. The mono- and bispecific responder macaques may not have had MHC class II molecules suitable for presenting immunizing TT 830-846 lipopeptide. This problem could be overcome in humans by using several promiscuous peptides that are presented by a majority of MHC class II molecules. Adding another T helper epitope or synthesizing more immunogenic lipopeptides containing modified peptides (25) may better stimulate a Th1 response for inducing anti-HIV multispecific CTL responses. HIV-specific Th epitopes would be more relevant to induce strong helper activity at the time of HIV infection. Few HIV Th epitopes have been described to date, but the restoration of anti-HIV proliferative responses (1, 13, 30) by highly active antiretroviral treatment of primary infected humans may provide a better definition of Th epitopic regions.

Finally, our findings suggest that anti-TT 830-846 T lymphocytes with a Th1 profile are most important and indicate that soluble factors like IFN-γ, and probably interleukin 2 (IL-2), help to induce optimal differentiation of CTL responses, leading to multispecific cytotoxic responses. These results appear to be promising for the future development of peptide vaccines to protect against HIV infection.

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