Long-Term Specific Immune Responses Induced in Humans by a Human Immunodeficiency Virus Type 1 Lipopeptide Vaccine: Characterization of CD8⁺-T-Cell Epitopes Recognized

Hanne Gahéry-Ségard,^{1*} Gilles Pialoux,² Suzanne Figueiredo,¹ Céline Igéa,¹ Mathieu Surenaud,¹ Jessintha Gaston,¹ Helene Gras-Masse,³ Jean-Paul Lévy,⁴ and Jean-Gérard Guillet¹

Département d'Immunologie-Membre de l'IFR 116-INSERM U567, Institut Cochin, 75014 Paris,¹ Département des Maladies Infectieuses, Hôpital Tenon, 75020 Paris,² UMR 8525 CNRS-Université Lille II, Institut Pasteur de Lille, 1, 59021 Lille,³ and Institut Pasteur, 75015 Paris,⁴ France

Received 27 March 2003/Accepted 29 July 2003

We studied the effect of booster injections and the long-term immune response after injections of an anti-human immunodeficiency virus type 1 (HIV-1) lipopeptide vaccine. This vaccine was injected alone or with QS21 adjuvant to 28 HIV-uninfected volunteers. One month later, after a fourth injection of the vaccine, B- and T-cell anti-HIV responses were detected in >85% of the vaccinated volunteers. One year after this injection, a long-term immune response was observed in >50% of the volunteers. At this point, a positive QS21 effect was observed only in the sustained B-cell and CD4⁺-T-cell responses. To better characterize the CD8⁺-T-cell response, we used a gamma interferon enzyme-linked immunospot method and a bank of 59 HIV-1 epitopes. For the six most common HLA molecules (HLA-A2, -A3, -A11, -A24, -B7 superfamily, and -B8), an average of 10 (range, 3 to 15) HIV-1 epitopes were tested. CD8⁺-T-cell responses were evaluated according to the HLA class I molecules of the volunteers. Each assessment was based on 18 HIV-1 epitopes in average. We showed that 31 HIV-1 epitopes elicited specific CD8⁺-T-cell responses after vaccination. The most frequently recognized peptides were Nef 68-76 (-B7), Nef 71-79 (-B7), Nef 84-92 (-A11), Nef 135-143 (-B7), Nef 136-145 (-A2), Nef 137-145 (-A2), Gag 259-267 (-B8), Gag 260-268 (-A2), Gag 267-274 (-A2), Gag 267-277 (-B7), and Gag 276-283 (A24). We found that CD8⁺-T-cell epitopes were induced at a higher number after a fourth injection ($P < 0.05$ compared to three injections), which indicates an increase in the breadth of HIV CD8⁺-T-cell epitope recognition after the boost.

In Europe and North America, an important decrease in mortality due to antiretroviral agents was observed among humans infected with human immunodeficiency virus type 1 (HIV-1). However, an HIV-1 vaccine offers the best long-term hope to control the AIDS pandemic, since the vast majority of individuals do not have access to these new treatment agents and new infection cases continue to occur (13).

There is growing evidence that cytotoxic T lymphocytes (CTLs) are an important component of the antiviral responses in both HIV-infected people and simian immunodeficiency virus (SIV)-infected macaques. Eliminating CD8⁺ lymphocytes from monkeys during chronic SIV infection resulted in a rapid and marked increase in viremia, which was again suppressed when SIV-specific CD8⁺ T cells reappeared (35). The involvement of CD8⁺ T cells in the clearance of the virus could explain the detection of CTL responses in exposed, noninfected people such as sex workers (18). Moreover, to explain why CTLs are unable in many cases to adequately control virus expansion in HIV infection, it has been shown that many of these cells do not seem to be functional, possibly because of impaired maturation, which leaves the patient with high numbers of nonfunctional virus-specific CD8⁺ T cells (4, 5, 7). These results confirm the importance of cell-mediated immu-

nity in controlling HIV-1 infection and support the exploration of infection-preventing vaccination approaches that will elicit these immune responses (25). Today, researchers from both HIV Vaccine Trials Network of the NIH and Agence Nationale de Recherche sur le SIDA (ANRS) consider that a phase III trial could be launched if a candidate vaccine was able to induce killer cells in at least 30% of vaccinated people included in a phase II trial (9, 22).

On the other hand, induction and maintenance of CD8⁺ T cells require specific CD4⁺ helper T lymphocytes. Virus CD4⁺ T cells have been shown to play an important role in maintaining effective CTL function and in controlling viremia during several chronic viral infections (17). Vigorous HIV-1 p24-specific CD4⁺ proliferative responses were more frequently found in the peripheral blood of HIV-1-infected patients with nonprogressive disease and were associated with control of viremia (29, 31).

To date, over 25 different HIV vaccines have been tested in human trials and have shown potential, but only a few of these trials focused on the induction of CTLs. Because epitope-based vaccines offer several potential advantages for inducing strong, multispecific CTL responses, we developed a multiepitopic HIV-1 vaccine based on lipopeptides. McMichael and coworkers studied the ability of a CD8⁺ epitope-based approach to induce CTL responses in rhesus monkeys by using DNA prime and modified vaccinia virus Ankara (MVA) boost vaccine. Direct ex vivo SIV-specific cytotoxic activity was detected in peripheral blood mononuclear cells (PBMCs) from

* Corresponding author. Mailing address: Département d'Immunologie-Institut Cochin, Hôpital Cochin, 27, rue du faubourg Saint-Jacques, 75014 Paris, France. Phone: 33(0)1-43-21-04-80. Fax: 33(0)1-40-48-83-52. E-mail: gahery@cochin.inserm.fr.

five of the six DNA/MVA-vaccinated animals (2, 16). Based on these results, recombinant DNA and MVA vaccines were tested in a phase I clinical trial in the United Kingdom and Kenya (15). A major difference with the vaccine described by Hanke et al. (16) is that our candidate vaccine is designed to include large synthetic fragments derived from different natural HIV-1 proteins and containing multipotential CD4⁺- and CD8⁺-T-cell epitopes that could be processed by appropriate humans cells (8, 14). The immune responses obtained after lipopeptide vaccination could be explained in part by the fact that the lipid moiety induces endocytosis of lipopeptides into dendritic cells and by the fact that the exogenous protein pathway can induce specific CD8⁺ T cells (3). Large synthetic peptide-based approaches present several advantages over conventional vaccine approaches (i.e., the use of proteins, whole DNA gene, and live recombinant vectors). The immune response can be directed against highly conserved epitopes that might be crucial for pathogens such as HIV. This approach also provides the ability to elicit CTL responses directed against subdominant epitopes and to eliminate epitopes that could induce deleterious immune responses.

Thus, several studies in mice (33), primates (6, 27), and humans (36) have shown that lipopeptides are highly immunogenic *in vivo*. The frequency and duration of the CTL response are directly influenced by the presence of potent CD4⁺-T-cell epitopes (23, 26).

We have shown previously that an anti-HIV lipopeptide vaccine by using large peptides derived from regulatory or structural HIV-1 proteins (Nef, Gag, and Env) injected alone or with QS21 adjuvant to HIV-uninfected volunteers is well tolerated and is able to induce immune responses already after three injections (14, 28). We analyzed here the effect of booster immunization after a fourth injection and the long-term immune response measured 2 years after the first injection of the vaccine.

From the vaccine, 59 HIV-1 CD8⁺-T-cell epitopes were identified and used with an gamma interferon (IFN- γ) enzyme-linked immunospot (ELISPOT) method to characterize the anti-HIV-1 CD8⁺-T-cell responses. We also evaluated the breadth of CD8⁺-T-cell responses induced after injection of the vaccine.

MATERIALS AND METHODS

Sequences and synthesis of lipopeptides. The synthesis, formulation, and the solubilization of the lipopeptide vaccines have been described previously (19). Three lipopeptides, L-N1 (Nef amino acid [aa] 66 to 97), L-N2 (Nef aa 117 to 147), and L-N3 (Nef aa 182 to 205), were derived from HIV-LAI Nef proteins; two lipopeptides, L-G1 (Gag aa 183 to 214) and L-G2 (Gag aa 253 to 284), were derived from HIV-LAI Gag proteins; and one lipopeptide, L-E (Env aa 303 to 335), was derived from an V3-Env gp120 consensus sequence that is also found in the HIV-BX08 strain. The amino acid sequences of the six lipopeptides are presented in Fig. 1.

Long and short peptides. The long peptides (N1, N2, N3, G1, G2, and E) corresponding to the lipopeptide immunogens were synthesized by Neosystem (Strasbourg, France). The 59 short peptides overlapping the large HIV-1 peptide sequences and known to be minimal CD8⁺-T-cell epitopes were also synthesized by Neosystem. Some sequences of these HIV-1 peptides can be found in the NIH molecular database and in recent publications (8, 11, 12; HIV Molecular Immunology Database [<http://hiv-web.lane.gov/immuno/index.html>]). Figure 1 presents the sequences of all CD8⁺-T-cell epitopes, and the HLA class I restrictions are indicated between brackets.

Immunization protocol and study design. Volunteers were selected by ANRS among populations that are not exposed to HIV. This trial was a phase I designed

to determine the clinical tolerance of the lipopeptide vaccine. Written consent was obtained from each volunteer; the nature and consequences of the studies were also explained. A classical clinical investigation was performed to determine the serology to HIV, as described in our previous studies (14, 28). In the first part of the trial, 28 volunteers were immunized three times with a mixture of six lipopeptides (half of the volunteers also received a QS21 adjuvant) injected intramuscularly at 0, 4, and 16 weeks (14, 28). The clinical tolerability was good, and the volunteers received a fourth injection of 500 μ g of each lipopeptide at week 48 (W48), except volunteers V4.8 and V4.4, who received only 250 μ g of each lipopeptide. We present here the immunological data from the second part of the trial. Blood samples were collected prior to immunization (at W0), after three immunizations (at W20), at W44, at W52 (4 weeks after the fourth injection), and at W104. PBMCs and sera were separated by standard methods and frozen. After immunization at W20 (after three injections), W44, W52 (after four injections), and W104, we checked for the induction of a specific immune response in vaccinated volunteers, and we took as our negative reference the same volunteer before immunization (W0).

Anti-HIV peptide proliferative T-cell responses. Fresh PBMCs and CD4⁺ or CD8⁺ T cells (10^5 cells/well) were cultured in complete medium with 1 μ g of one of the soluble peptides (N1, N2, N3, G1, G2, and E)/ml. The experiment was set up in quadruplicate. Proliferation was determined in culture on day 7 by adding 1 μ Ci of [³H]thymidine (NEN, Paris, France)/well. The capacity of the PBMCs and CD4⁺ or CD8⁺ T cells to proliferate *in vitro* was controlled in independent cultures carried out for 7 days with phytohemagglutinin (PHA; 4 μ g/ml), purified protein derivative (1 μ g/ml), tetanus toxoid (1 μ g/ml), and staphylococcal enterotoxin B (0.1 μ g/ml).

Identification of CD8⁺ T cells by IFN- γ ELISPOT assay. A one-step stimulation strategy was used in this assay to amplify the CD8⁺-T-cell responses before ELISPOT assay. We incubated cryopreserved PBMCs (4×10^6 cells) for 2 h at 37°C in complete medium (RPMI 1640 supplemented with 10% human AB serum) with 1 μ g of each CD8⁺-T-cell epitope per ml (five to six peptides at most were mixed). We also used as a positive-control HLA-matched peptides derived from Epstein-Barr virus, influenza virus, and cytomegalovirus. The cells were washed twice in RPMI 1640 with 3% SAB and then incubated in complete medium (2×10^5 cells/ml) at 37°C with 5% CO₂. We added to the cultured cells 10 U of interleukin-2 (Boehringer)/ml at 3 and 7 days. Finally, the cells were collected at day 12 for an IFN- γ ELISPOT assay. For this assay, 96-well nitrocellulose plates (MultiScreen-HA; Millipore S.A., Molsheim, France) were coated with 0.1 μ g of mouse anti-human MAb (1-D1K; Mabtech, Nacka, Sweden) per well and left overnight at 4°C. The wells were washed in phosphate-buffered saline and saturated with complete RPMI medium. The HIV-1 peptides (10 μ g/ml) were added to the corresponding 12-day cell cultures (10^5 cells/well), and the mixture was incubated 5 h at 37°C with 5% CO₂. The plates were then washed and incubated overnight with 0.1 μ g of an biotinylated monoclonal anti-human IFN- γ antibody (7-B6-1; Mabtech) per well. Finally, alkaline phosphatase-labeled extravidin (Sigma-Aldrich Chimie SARL), diluted at 1/6,000, was added for 1 h. A total of 100 μ l of chromogenic alkaline phosphatase substrate (Bio-Rad Laboratories) was added to each well to develop spots. Blue spots were counted with an automatic microscope (Zeiss Apparatus; Carl Zeiss, Göttingen, Germany). Negative controls consisted of PBMCs incubated in medium alone or with HLA-mismatched CD8⁺-T-cell epitopes derived from HIV-1 virus. Positive controls consisted of the activation of PBMCs with 1 μ g of PHA/ml (25,000 cells per well).

Statistical analyses. Data analyses were performed with the StatView 5.0 software (Abacus Concepts, San Francisco, Calif.). Comparisons between variables were performed by using analysis of variance or Mann-Whitney test. *P* values of ≤ 0.05 were considered significant.

RESULTS

The 28 volunteers included in ANRS VAC 04 trial completed the immunization schedule (four immunizations). The details of the study protocol are presented in Materials and Methods. The vaccine was well tolerated after four injections, as already reported after three injections (28). The complete kinetics of the T-cell immune response induced was detailed for the 28 HIV-seronegative volunteers.

Booster immunization effect and sustained lymphoproliferative responses specific to HIV-1 long peptides. Proliferative responses to soluble Nef, Gag, and Env long peptides obtained

FIG. 1. HLA-restricted HIV-1 peptides derived from the six lipopeptides. (A) HLA-restricted HIV-1 peptides in the three Nef regions. These regions have the potential to generate many peptides that can react with different MHCs (HLA restriction is identified between brackets). The names and positions of each peptide are presented. B7# indicates superfamily B7, including HLA-B7, -B35, -B51, and B53-restricted peptides. \$. Two Nef 71-81 peptides with the HLA-B7 motif were derived: Nef 71-81, with an R in position 71, is a variant of Nef 71-81 from Nef HIV-1/LAI

with PBMCs from the 28 vaccinated subjects are detailed in Tables 1 and 2. No proliferation was found prior to vaccination, as described in another study (28). The PBMCs of the majority of volunteers immunized with or without QS21 adjuvant induced a proliferation against at least one peptide after three injections (Tables 1 and 2). At W44, the proliferative response was still present in the majority of the volunteers tested (data not shown). After the fourth injection, at W52, a proliferative response to at least one peptide was seen in 24 of the 26 volunteers tested, but the increase in the number of positive responders was not significant, which made it impossible to reach a definite conclusion concerning the boost ($P > 0.05$ compared to W20). Proliferation of PBMCs in response to N1 was observed in 6 of the 26 volunteers tested, with proliferative indexes (relative to PBMCs at W0) varying from 3.2 to 9.9. The proliferative response to N2 was positive in 12 of the 26 volunteers tested. Finally, proliferation in response to N3 long peptide was observed with PBMCs from 13 of the 26 vaccinated volunteers. PBMCs collected from the volunteers were also assayed for proliferation in response to G1 and G2 long peptides. The PBMCs from 25 of 26 volunteers remained negative to G1 peptides after four injections, proliferation in response to G1 peptide was detected in only one volunteer immunized with QS21 adjuvant (V4.19-QS21). In contrast, proliferation in response to G2 long peptide was determined with PBMCs from 20 of the 26 vaccinated subjects, with proliferative indexes varying from 3.7 to 45. Specific proliferative responses to the E long peptide were observed with PBMCs from 11 of the 26 volunteers. The number of Nef, Gag, and Env CD4⁺-T-cell peptides recognized at W52 were not statistically different from those observed at W20 ($P > 0.05$). Finally, a long-lasting proliferative response against at least one peptide was observed at W104 in 14 of the 21 volunteers tested. The long-lasting proliferative response was mainly against G2 long peptide, since PBMCs from 13 of the 14 positive responders proliferated in response to this peptide. The sustained multispecific lymphoproliferative response was found in all of the 11 tested volunteers immunized with QS21 adjuvant. The percentage of positive responders in this group was significantly higher ($P = 0.007$ compared to the group immunized with the lipopeptide vaccine alone).

A depletion experiment was carried out with PBMCs from 7 of the 12 vaccinated subjects at W104. Cell proliferation assays performed in the presence of HIV-1 long peptides with PBMCs and CD4⁺ or CD8⁺ T cells showed that proliferation was preferentially mediated by CD4⁺ T cells (data not shown).

Booster immunization effect and sustained HIV-specific CD8⁺-T-cell response. The IFN- γ ELISPOT assay appeared to be a very sensitive method for identifying CD8⁺ T cells. We have already reported that PBMCs from some volunteers rec-

ognized HIV-1 CD8⁺-T-cell peptides, as evidenced by an ex vivo ELISPOT assay, whereas no CTL activity against the HIV-1 long peptides was identified (14). However, by using the ex vivo ELISPOT to study the CD8⁺-T-cell responses in HIV-seronegative vaccinated volunteers, we have shown that this method was not completely appropriate because the number of activated effector CD8⁺ T cells was very low and some time borderline. Russell et al. (32) confirmed this point by showing borderline CD8⁺-T-cell responses after an ex vivo ELISPOT evaluation in HIV-1-uninfected vaccinated volunteers. (These authors detected 2 to 50 spot-forming cells [SFCs]/10⁶ of cells.) Therefore, we decided to use the ELISPOT method combined with a one-step stimulation before the assays. This stimulation method was derived from the classical international chromium release test previously used to measure CD8⁺ T cells in all vaccine trials in healthy volunteers. All of these previous trials showed that repeated stimulations were necessary for detection of CTL activity. Our objective in the present trial was to characterize CD8⁺-T-cell prime-precursors (i.e., the breadth of CD8⁺-T-cell responses, the long-term immune response, and identification of the CD8⁺-T-cell epitopes induced after vaccination). To characterize the CD8⁺-T-cell responses induced after vaccination, we stimulated the PBMCs from the vaccinated volunteers once with a bank of optimum CD8⁺-T-cell epitopes and used the IFN- γ ELISPOT assay to detect the specific responses. The optimum CD8⁺ T cells (59 HIV-1 peptides) derived from the six HIV-1 lipopeptides (Fig. 1) were incubated with PBMCs according to the HLA class I molecules identified from volunteers (Tables 3 and 4).

For all of the volunteers, the kinetics of CD8⁺-T-cell induction was characterized in the same experiment, and positive CD8⁺-T-cell responses were confirmed at least one more time. Because there was no placebo group in this phase I trial, we defined a CD8⁺-T-cell response induction for a given peptide when the PBMCs from the same volunteer were negative before immunization (W0). The blood sample collected prior to immunization (W0) was taken as a negative reference in the present study and tested at the same time as the blood samples collected after immunization (W20, W44, W52, and W104). Moreover, Russell et al. (32) showed that cryopreservation had no significant effect on the number of SFCs detected when these authors compared results with fresh and cryopreserved cells. Thus, in the present study, we used cryopreserved PBMCs to test the CD8⁺-T-cell induction in presence of specific HIV-1 CD8⁺-T-cell epitopes and, by studying non-HIV viral CD8⁺ peptides derived from Epstein-Barr virus, cytomegalovirus, and influenza virus, we estimated the functionality of frozen PBMCs (data not shown).

Tables 3 and 4 present the complete results of the CD8⁺-T-cell study, performed with PBMCs from the volunteers

sequence with a T in position 71. *, Two Nef 82-91 peptides with an HLA-A2/B51 motif were derived: Nef 82-91, with an L in position 85, is a variant of Nef 82-91 from Nef HIV-1/LAI sequence with a V in position 85. ‡, Two 190-198 peptides were derived: Nef 190-198, with an L in position 191, has an HLA-A2 motif and is a variant of Nef 190-198 from Nef HIV-1/LAI sequence with an F in position 191 and with an HLA-A24 motif. (B) HLA-binding epitopes in the two Gag regions (G1 and G2) and in the Env region (E). The names and the positions of the potential Gag and Env epitopes are presented. ¥, Two Gag 267-277 peptides with a B7 motif were derived: Gag 267-277 with a V in position 268, is a variant of Gag 267-277 from Gag HIV-1/LAI sequence with an L in position 268. Finally, HIV-1 peptides that did not elicit a CD8⁺-T-cell response after vaccination are represented by a dotted line. HIV-1 peptides that elicited a specific anti-HIV CD8⁺-T-cell response are indicated by a bold line. The thick line identifies the HIV-1 peptides that were more frequently recognized.

TABLE 1. Proliferative responses of PBMC from volunteers immunized with lipopeptide alone^a

Volunteer	Wk	Mean stimulation index $\pm$ SD in response to:						Medium (mean cpm $\pm$ SD)
		N1	N2	N3	G1	G2	E	
V4.3	W20	5.8 $\pm$ 0.5	—	7.0 $\pm$ 1.9	—	—	—	1,046 $\pm$ 110
	W52	—	—	3.7 $\pm$ 0.2	—	—	—	13,469 $\pm$ 2,092
	W104	ND	ND	ND	ND	ND	ND	ND
V4.6	W20	—	—	10.0 $\pm$ 7	4.5 $\pm$ 1	7.2 $\pm$ 0.7	—	280 $\pm$ 32
	W52	—	6.5 $\pm$ 3.3	8.0 $\pm$ 5.3	—	10.0 $\pm$ 1.7	—	403 $\pm$ 22
	W104	—	—	—	—	—	—	3,207 $\pm$ 1,678
V4.8	W20	9.3 $\pm$ 3.8	9.9 $\pm$ 3.5	—	—	—	—	599 $\pm$ 39
	W52	3.2 $\pm$ 0.1	7.9 $\pm$ 1.7	—	—	9.8 $\pm$ 2.5	7.4 $\pm$ 2.2	2,700 $\pm$ 458
	W104	—	—	—	—	—	—	740 $\pm$ 222
V4.12	W20	—	—	—	—	9.1 $\pm$ 1.9	—	1,381 $\pm$ 75
	W52	—	20 $\pm$ 1.8	—	—	4.6 $\pm$ 0.9	—	450 $\pm$ 85
	W104	—	—	—	—	—	—	3,497 $\pm$ 1,081
V4.15	W20	ND ^b	—	—	ND	ND	—	252 $\pm$ 30
	W52	—	9.7 $\pm$ 3.8	—	—	34.5 $\pm$ 7.1	12.7 $\pm$ 5.8	531 $\pm$ 106
	W104	—	—	—	—	13.4 $\pm$ 8.4	—	1,559 $\pm$ 809
V4.16	W20	—	—	—	4.6 $\pm$ 0.6	3.6 $\pm$ 0.5	3.9 $\pm$ 0.7	1,000 $\pm$ 168
	W52	—	—	25.0 $\pm$ 8.0	—	3.7 $\pm$ 0.5	55.0 $\pm$ 10	171 $\pm$ 17
	W104	ND	ND	ND	ND	ND	ND	ND
V4.17	W20	—	—	—	—	—	—	1,228 $\pm$ 54
	W52	9.9 $\pm$ 5.8	4.9 $\pm$ 1.2	—	—	—	—	164 $\pm$ 8
	W104	—	—	—	—	—	—	2,934 $\pm$ 757
V4.18	W20	ND	ND	ND	ND	ND	ND	ND
	W52	—	—	—	—	6.8 $\pm$ 1.9	—	509 $\pm$ 45
	W104	—	—	—	—	—	—	2,693 $\pm$ 671
V4.20	W20	9.3 $\pm$ 1.3	—	ND	ND	—	—	2,353 $\pm$ 385
	W52	—	—	5.3 $\pm$ 0.4	—	3.9 $\pm$ 0.3	—	18,771 $\pm$ 1,745
	W104	ND	ND	ND	ND	ND	ND	ND
V4.23	W20	—	—	—	—	14.9 $\pm$ 3.9	3.6 $\pm$ 0.5	926 $\pm$ 60
	W52	—	—	—	—	—	6.2 $\pm$ 3.1	789 $\pm$ 75
	W104	—	—	—	—	5.8 $\pm$ 1.8	—	2,060 $\pm$ 709
V4.26	W20	17.6 $\pm$ 9.0	—	27.0 $\pm$ 9.0	—	—	—	436 $\pm$ 56
	W52	—	—	—	—	—	—	591 $\pm$ 72
	W104	—	—	—	—	—	—	985 $\pm$ 441
V4.28	W20	3.8 $\pm$ 0.6	—	21.0 $\pm$ 20	—	8.2 $\pm$ 1.6	7.6 $\pm$ 2.2	2,558 $\pm$ 186
	W52	3.3 $\pm$ 0.1	—	4.9 $\pm$ 1.6	—	16.0 $\pm$ 3.2	10.7 $\pm$ 3.3	512 $\pm$ 42
	W104	—	—	8.0 $\pm$ 4.6	—	21.1 $\pm$ 5.5	—	2,170 $\pm$ 1,199
V4.30	W20	—	—	—	—	—	—	9,535 $\pm$ 469
	W52	—	—	—	—	—	—	312 $\pm$ 58
	W104	—	—	—	—	—	—	4,130 $\pm$ 2,479

^a A total of 10⁵ PBMCs collected after injection of the vaccine (at W20, W52, and W104) were cultured with 1 μ g of HIV long peptides (N1, N2, N3, G1, G2, or E). The proliferative response of PBMCs cultured in medium alone is given in the right-hand column in counts per minute. The stimulation index obtained with culture medium alone is taken as 1. Means of four replicates are expressed in counts per minute. A positive index is defined as an index of $>3 \pm$ the standard deviation. A negative proliferation (<3) is identified by a minus sign (—). Proliferative response was not determined for V4.14. ND, not done.

tested. The kinetics of CD8⁺-T-cell induction was evaluated by testing PBMCs collected before vaccination (W0) and after vaccination at W20, W44, W52, and W104 in 17, 22, 22, and 23 vaccinated volunteers, respectively. For each volunteer, an average of 18 (range, 3 to 39) HIV-1 peptides were tested. In 12 of the 17 volunteers tested at W20, PBMCs were able to secrete IFN- γ in response to at least one HIV-1 peptide. Of the 12 positive volunteers, 7 induced a polyspecific CD8⁺-T-cell

response. The positive CD8⁺-T-cell response at W44 was not significantly lower than that observed at W20 ($P > 0.05$). At that time, 10 (58.8%) of the 17 positive volunteers tested gave a monoepitopic CD8⁺-T-cell response. The effect of the boost injection at W48 was tested at W52. We found that 86% of the volunteers gave a positive CD8⁺-T-cell response, and 13 (68.4%) of the 19 positive volunteers recognized at least two different HIV-1 peptides, but this percentage was not signifi-

TABLE 2. Proliferative responses of PBMC from volunteers immunized with lipopeptides and QS21 adjuvant

Volunteer	Wk	Mean stimulation index $\pm$ SD in response to ^a :						Medium (mean cpm $\pm$ SD)
		N1	N2	N3	G1	G2	E	
V4.1-QS	W20	ND	ND	ND	ND	ND	ND	ND
	W52	3.9 $\pm$ 0.4	—	3.9 $\pm$ 0.2	—	7.9 $\pm$ 0.5	—	9,610 $\pm$ 441
	W104	—	—	—	—	14.5 $\pm$ 3.4	—	5,148 $\pm$ 812
V4.2-QS	W20	—	—	—	—	—	—	715 $\pm$ 46
	W52	8.3 $\pm$ 0.5	—	11.6 $\pm$ 3.7	—	16.0 $\pm$ 4.2	4.6 $\pm$ 1.5	974 $\pm$ 63
	W104	6.1 $\pm$ 2.3	—	35.6 $\pm$ 16	—	63.7 $\pm$ 7.1	20.1 $\pm$ 8.8	1,576 $\pm$ 308
V4.4-QS	W20	—	—	—	—	—	—	510 $\pm$ 68
	W52	—	—	—	—	6.1 $\pm$ 2.5	—	2,006 $\pm$ 131
	W104	—	—	—	—	3.8 $\pm$ 0.5	—	3,056 $\pm$ 796
V4.5-QS	W20	4.6 $\pm$ 1.2	—	—	—	3.9 $\pm$ 0.3	5.0 $\pm$ 2.2	776 $\pm$ 60
	W52	—	4.2 $\pm$ 0.6	7.0 $\pm$ 3.5	—	5.6 $\pm$ 2	—	220 $\pm$ 18
	W104	—	—	—	—	7.5 $\pm$ 3.3	—	379 $\pm$ 91
V4.11-QS	W20	—	—	6.1 $\pm$ 0.4	5.9 $\pm$ 0.1	6.6 $\pm$ 1.5	7.0 $\pm$ 1.9	3,938 $\pm$ 970
	W52	—	5.6 $\pm$ 1.5	9.1 $\pm$ 4.5	—	5.6 $\pm$ 1.2	6.4 $\pm$ 2.3	3,210 $\pm$ 348
	W104	8 $\pm$ 4.2	—	22.3 $\pm$ 4.3	6.2 $\pm$ 3.1	14.5 $\pm$ 3.5	18.1 $\pm$ 5.9	1,613 $\pm$ 200
V4.13-QS	W20	11.5 $\pm$ 2.7	—	9.3 $\pm$ 2.0	—	15.0 $\pm$ 2.0	—	812 $\pm$ 77
	W52	—	5.0 $\pm$ 0.4	7.9 $\pm$ 0.9	—	22.2 $\pm$ 4.6	—	1,724 $\pm$ 251
	W104	7.4 $\pm$ 1.3	—	4.1 $\pm$ 1.1	—	34.3 $\pm$ 2.8	—	3,264 $\pm$ 270
V4.19-QS	W20	24.3 $\pm$ 3.1	8.5 $\pm$ 5.0	4.4 $\pm$ 1.2	—	11.0 $\pm$ 2.7	9.4 $\pm$ 2.8	497 $\pm$ 168
	W52	—	7.1 $\pm$ 2.8	6.6 $\pm$ 0.6	4.0 $\pm$ 0.8	11.1 $\pm$ 0.8	9.0 $\pm$ 0.9	7,474 $\pm$ 963
	W104	18.2 $\pm$ 10.0	—	—	—	—	4.9 $\pm$ 0.1	668 $\pm$ 314
V4.21-QS	W20	6.5 $\pm$ 3.0	—	—	—	11.3 $\pm$ 4.0	—	1,052 $\pm$ 82
	W52	—	5.6 $\pm$ 0.6	—	—	5.7 $\pm$ 2.0	—	2,114 $\pm$ 190
	W104	ND	ND	ND	ND	ND	ND	ND
V4.25-QS	W20	6.6 $\pm$ 2.3	14.6 $\pm$ 6.0	—	6.2 $\pm$ 1.6	—	—	592 $\pm$ 81
	W52	ND	ND	ND	ND	ND	ND	ND
	W104	70.0 $\pm$ 28.0	—	—	—	54.0 $\pm$ 24.0	60.0 $\pm$ 16.0	921 $\pm$ 606
V4.27-QS	W20	—	—	—	—	4.1 $\pm$ 0.3	—	1,978 $\pm$ 225
	W52	—	5.5 $\pm$ 1.1	—	—	4.2 $\pm$ 1.0	8.1 $\pm$ 1.9	2,410 $\pm$ 285
	W104	—	18 $\pm$ 4.4	—	—	43.6 $\pm$ 13.5	10.5 $\pm$ 5.9	1,889 $\pm$ 755
V4.29-QS	W20	—	—	—	—	—	—	2,543 $\pm$ 133
	W52	—	—	—	—	—	8.6 $\pm$ 2.6	738 $\pm$ 103
	W104	—	—	32.6 $\pm$ 9.5	—	13.5 $\pm$ 5.8	5.7 $\pm$ 1.9	1,466 $\pm$ 323
V4.32-QS	W20	—	—	—	—	10.1 $\pm$ 1.5	—	245 $\pm$ 30
	W52	—	4.6 $\pm$ 0.3	77.0 $\pm$ 12.0	—	45 $\pm$ 7.1	51.0 $\pm$ 5.2	407 $\pm$ 37
	W104	—	—	—	—	6.2 $\pm$ 2.7	—	4,887 $\pm$ 1,154
V4.33-QS	W20	—	—	—	—	7.3 $\pm$ 2.6	—	218 $\pm$ 9
	W52	—	—	—	—	7.8 $\pm$ 5.0	—	240 $\pm$ 39
	W104	ND	ND	ND	ND	ND	ND	ND
V4.34-QS	W20	3.4 $\pm$ 0.2	—	3.3 $\pm$ 0.1	—	4.4 $\pm$ 0.6	3.1 $\pm$ 0.1	7,381 $\pm$ 280
	W52	4.4 $\pm$ 0.4	—	3.5 $\pm$ 0.2	—	3.6 $\pm$ 0.2	—	11,250 $\pm$ 570
	W104	ND	ND	ND	ND	ND	ND	ND

^a See Table 1, footnote a.

cantly higher than that observed at W20 ($P > 0.05$). However, a higher number of different CD8⁺-T-cell epitopes were induced at W52 ($P = 0.0006$ compared to week 20 and $P < 0.0001$ compared to W44), which indicates an increase in the breadth of HIV CD8⁺-T-cell epitopes induced after the boost. Finally, a sustained CD8⁺-T-cell response was detected at W104 in 13 of 23 volunteers tested. A sustained multispecific

CD8⁺-T-cell response was found in four of the seven positive volunteers immunized with lipopeptide alone. Four of the six positive volunteers from the group immunized with QS21 adjuvant presented this sustained polyspecific CD8⁺-T-cell response. However, the effect of this adjuvant on the sustained CD8⁺-T-cell response was not statistically significant ($P > 0.05$ compared to the group without adjuvant).

TABLE 3. CD8⁺-T-cell peptides induced in volunteers after immunization with lipopeptides alone

Volunteer and HLA type(s) ^a (no. of HIV peptides tested)	HIV (HLA) positive peptides ^b	Mean no. of SFCs/million $\pm$ SD ^c at:				
		W0	W20	W44	W52	W104
V4.3 and HLA-A2, -B18/B58 (15 HIV-1)	Gag 260-268 (-A2)	—	1,790 $\pm$ 125	—	390 $\pm$ 85	995 $\pm$ 95
	Gag 267-274 (-A2)	—	—	—	420 $\pm$ 5	715 $\pm$ 45
	Gag 275-283 (-A2)	—	1,320 $\pm$ 120	505 $\pm$ 150	1,080 $\pm$ 25	615 $\pm$ 115
V4.6 and HLA-A2/A68, -B39/B53 (38 HIV-1)	Nef 68-76 (-B53)	—	1,190 $\pm$ 170	1,360 $\pm$ 80	1,750 $\pm$ 90	360 $\pm$ 110
	Nef 135-143 (-B53)	—	2,015 $\pm$ 35	650 $\pm$ 75	1,593 $\pm$ 300	590 $\pm$ 210
	Gag 267-277 (-B53)	—	520 $\pm$ 60	330 $\pm$ 55	1,560 $\pm$ 485	—
V 4.8 and HLA-A3/A24, -B7/B27 (39 HIV-1)	Gag 267-277 (-B7)	—	—	—	1,250 $\pm$ 390	—
	Gag 276-283 (-A24)	—	860 $\pm$ 455	660 $\pm$ 455	615 $\pm$ 30	365 $\pm$ 30
	Gag 263-272 (-B27)	—	2,880 $\pm$ 150	346 $\pm$ 37	440 $\pm$ 5	—
V 4.12 and HLA-A3/11, -B8 (7 HIV-1)	Nef 84-92 (-A3/A11)	—	ND	ND	840 $\pm$ 340	—
V 4.14 and HLA-A1/A2, -B15/B57 (20 HIV-1)	Nef 121-128 (-A1)	—	—	—	1,340 $\pm$ 130	—
	Nef 136-145 (-A2)	—	1,220 $\pm$ 35	—	1,360 $\pm$ 385	385 $\pm$ 140
	Nef 137-145 (-A2)	—	—	—	1,185 $\pm$ 285	—
	Gag 267-274 (-A2)	—	—	1,115 $\pm$ 250	—	—
V 4.15 and HLA-A3/A29, -B44/B53 (23 HIV-1)	Gag 266-275 (-A3)	—	ND	220 $\pm$ 25	265 $\pm$ 45	—
	Nef 71-79 (-B53)	—	ND	—	240 $\pm$ 5	—
	Nef 135-143 (-B53)	—	ND	315 $\pm$ 55	2,080 $\pm$ 230	850 $\pm$ 85
	Gag 254-262 (-B53)	—	ND	—	235 $\pm$ 90	—
	Gag 267-277* (-B53)	—	ND	—	455 $\pm$ 200	710 $\pm$ 45
	Gag 267-277 (-B53)	—	ND	390 $\pm$ 55	345 $\pm$ 90	1,445 $\pm$ 20
V 4.20 and HLA-A1/A24, -B7/B38 (27 HIV-1)	Nef 195-202 (-A1)	ND	1,555 $\pm$ 120	—	—	—
	Gag 276-283 (-A24)	ND	—	285 $\pm$ 16	780 $\pm$ 90	720 $\pm$ 5
V4.23 and HLA-A29/A32, -B51/35 (19 HIV-1)	Nef 68-76 (-B51/B35)	—	1,075 $\pm$ 15	535 $\pm$ 40	490 $\pm$ 260	—
	Nef 71-79 (-B51/B35)	—	1,375 $\pm$ 100	—	655 $\pm$ 130	—
	Nef 71-81 (-B51/B35)	—	—	1,560 $\pm$ 5	565 $\pm$ 90	—
V 4.26 and HLA-A29/A32, -B14 (3 HIV-1)	Nef 196-203 (-A29)	—	800 $\pm$ 0	—	320 $\pm$ 100	—
	Nef 133-141 (-A32)	—	—	690 $\pm$ 0	575 $\pm$ 0	—
V 4.28 and HLA-A1/6, -B5 (3 HIV-1)	—	—	—	—	—	—
V 4.30 and HLA-A2/-, -B61/64 (17 HIV-1)	Nef 82-91** (-A2)	—	—	—	1,810 $\pm$ 300	890 $\pm$ 315
	Nef 82-91 (-A2)	—	1,375 $\pm$ 115	2,065 $\pm$ 90	945 $\pm$ 325	2,475 $\pm$ 45
	Nef 83-91 (-A2)	—	1,212 $\pm$ 100	800 $\pm$ 30	980 $\pm$ 10	1,945 $\pm$ 135
	Nef 84-91 (-A2)	—	3,560 $\pm$ 340	1,575 $\pm$ 95	415 $\pm$ 10	2,030 $\pm$ 310
	Nef 190-198*** (-A2)	—	1,000 $\pm$ 45	1,005 $\pm$ 40	—	700 $\pm$ 170
	Nef 136-145 (-A2)	—	—	—	1,640 $\pm$ 140	685 $\pm$ 30
	Nef 137-145 (-A2)	—	—	—	1,595 $\pm$ 55	—
	Gag 260-268 (-A2)	—	—	—	1,605 $\pm$ 45	—

^a HLA class I molecules and number of HIV-1 CD8⁺-T-cell epitopes tested by ELISPOT assay are indicated for each volunteer. PBMCs from V4.16, V4.17, and V4.18 could not be tested (not enough cells).

^b Only positive HIV-1 CD8⁺-T-cell epitopes detected at least one time after immunization are listed. Gag 267-277* has a V in position 268. Nef 82-91** has an L in position 85. Nef 190-198*** has an L in position 191 and has an HLA-A2 motif.

^c Means (duplicates) are expressed as SFCs per million PBMCs after subtraction of the background measured with PBMCs alone. The SFCs/million value was calculated as follows: number of SFCs in stimulating wells (cells + peptide) – number of SFCs in unstimulating wells (cells) $\times$ 10. The response to a given peptide was considered positive when the stimulating well gave a differential greater than at least 200 IFN- γ SFCs/million from the unstimulated well. —, negative response; ND, not done.

We tested the possibility that successive immunization with lipopeptides could generate CD8⁺ T cells able to recognize HIV-1-mutated peptides by cross-reaction. The T-cell receptor (TCR) avidity for a given peptide could be modified by antigenic restimulation. Thus, we selected two peptides, Gag 267-277 and Nef 82-91, with one amino acid substitution. The CD8⁺ T cells from volunteers V4.15 and V4.32-QS21 were tested for their capacity to recognize the two different Gag 267-277 peptides. Tables 3 and 4 show that

CD8⁺ T cells from these two volunteers were able to recognize the two peptides. For volunteer V4.15, only the wild-type Gag 267-277 peptide elicited a response before the boost at W44, whereas both wild-type and mutant peptides elicited a response after the boost at W52. The same is true for the Nef 82-91 response in volunteer V4.30, since the wild-type Nef 82-91 peptide elicited a response both before the boost (W20) and after the boost (W52), whereas the mutant induced a specific response only after the boost

TABLE 4. CD8⁺-T-cell peptides induced in volunteers after immunization with lipopeptides and QS21

Volunteer and HLA type(s) ^a (no. of HIV peptides tested)	HIV (HLA) positive peptides ^b	Mean no. of SFCs/million $\pm$ SD ^c at:				
		W0	W20	W44	W52	W104
V 4.2-QS21 and HLA-A23/68, -B49/53 (22 HIV-1)		—	—	—	—	—
V 4.4-QS21 and HLA-A24/-, -B41/62 (14 HIV-1)	Gag 261-270 (-A24)	—	—	1,255 $\pm$ 10	490 $\pm$ 5	205 $\pm$ 5
V 4.5-QS21 and HLA-A2/11, -B18/27 (19 HIV-1)	Gag 263-272 (B27)	ND	ND	1,235 $\pm$ 90	735 $\pm$ 65	615 $\pm$ 45
	Nef 137-145 (-A2)	ND	ND	—	630 $\pm$ 185	490 $\pm$ 215
	Nef 84-92 (A11)	ND	ND	—	630 $\pm$ 95	615 $\pm$ 80
V 4.11-QS21 and HLA-A3/29, -B35/44 (11 HIV-1)		—	—	—	—	—
V 4.13-QS21 and HLA-A2/A24, -B51/B53 (31 HIV-1)	Nef 137-145 (-A2)	—	—	—	460 $\pm$ 50	—
	Nef 80-87 (-A24)	—	—	—	1,140 $\pm$ 100	220 $\pm$ 10
	Nef 134-143 (-A24)	—	1,175 $\pm$ 0	740 $\pm$ 100	850 $\pm$ 100	750 $\pm$ 30
	Gag 276-283 (-A24)	—	—	—	800 $\pm$ 40	485 $\pm$ 75
	Nef 128-135 (-B51/53)	—	—	—	1,220 $\pm$ 40	—
	Nef 135-143 (-B51/53)	—	530 $\pm$ 35	—	1,080 $\pm$ 370	400 $\pm$ 35
V 4.21-QS21 and HLA-A1/A32, -B8/B51 (23 HIV-1)	Gag 259-267 (-B8)	—	1,640 $\pm$ 80	760 $\pm$ 140	1,115 $\pm$ 445	—
V 4.25-QS21 and HLA-A2/A32, -B35/B44 (16 HIV-1)	Nef 135-143 (-B35)	—	ND	345 $\pm$ 20	ND	—
V 4.27-QS21 and HLA-A2/26, -B7/B51 (34 HIV-1)	Nef 137-145 (-A2)	—	ND	2,580 $\pm$ 275	1,470 $\pm$ 430	462 $\pm$ 108
	Gag 267-274 (-A2)	—	ND	—	625 $\pm$ 125	—
	Nef 128-137 (-B7/B51)	—	ND	—	2,012 $\pm$ 96	—
	Gag 267-277 (-B7/B51)	—	ND	785 $\pm$ 15	485 $\pm$ 235	280 $\pm$ 5
V 4.29-QS21 and HLA-A1/A3, -B8/- (9 HIV-1)	Gag 259-267 (-B8)	—	ND	—	1,610 $\pm$ 100	1,855 $\pm$ 590
V 4.32-QS21 and HLA-A26/68, -B35/51 (14 HIV-1)	Gag 267-277* (-B35/51)	—	6,490 $\pm$ 50	4,325 $\pm$ 35	615 $\pm$ 15	2,015 $\pm$ 115
	Gag 267-277 (-B35/51)	—	3,110 $\pm$ 130	5,765 $\pm$ 45	5,575 $\pm$ 55	2,880 $\pm$ 140
V 4.33-QS21 and HLA-A3, -B8/B65 (13 HIV-1)	Nef 182-189 (-B8)	—	—	—	430 $\pm$ 10	—
	Gag 259-267 (-B8)	—	1,235 $\pm$ 15	995 $\pm$ 50	730 $\pm$ 25	—
V 4.34-QS21 and HLA-A3/26, -B39/B60 (7 HIV-1)	Gag 267-275 (-A3)	—	—	—	495 $\pm$ 175	—

^a PBMC from volunteers V4.1-QS21 and V4.19-QS21 could not be tested. For other details, see Table 3, footnote *a*.

^b See Table 3, footnote *b*.

^c See Table 3, footnote *c*.

(W52). These results could indicate that increased avidity of the TCR for a wild-type peptide could allow recognition of a mutated peptide by cross-reaction.

Breadth the CD8⁺-T-cell epitopes induced in vaccinated volunteers. HLA-restricted CD8⁺-T-cell responses were evaluated with a wide range of synthetic HIV-1 peptides in 23 of the 28 volunteers. Of the 59 HIV-1 CD8⁺-T-cell epitopes tested, 31 induced stimulation (Fig. 1 [indicated by a thick line]). For the six common HLA molecules: (HLA-A2, -A3, -A11, -A24, -B7 superfamily, and -B8), an average of 10 (range, 3 to 15) HIV-1 peptides were tested per volunteer. Of the 15 HIV-1 peptides that can bind HLA-A2 molecule, 9 induced an anti-HIV-1 CD8⁺-T-cell response. Of these nine CD8⁺-T-cell epitopes, four (indicated in boldface in Table 5) were more frequently induced. There was an anti-HIV-specific response in six of eight (75%) vaccinated volunteers presenting the HLA-A2 molecule. In contrast, only three of the ten HLA-A3/A11-restricted peptides were recognized. Of these peptides, Nef 84-92 was identified in HLA-A11 vaccinated volunteers and the two others, Gag 266-275 and Gag 267-275, were identified in HLA-A3 volunteers. Of the eight HLA-A3- or -A11-vaccinated volunteers, four (50%) of them recognized specif-

ically at least one of these three peptides. All of the four HLA-A24-vaccinated volunteers tested, responded to at least one HIV-1 peptide. Four of the eight CD8⁺-T-cell epitopes restricted by the HLA-A24 molecule were recognized at different stages after vaccination. The Gag 276-283 peptide (HLA-A24) was identified in three of four volunteers tested. A total of 13 peptides restricted by HLA-B7 superfamily were tested in 12 volunteers. Of the 13 peptides, 8 elicited a positive response, and 66.6% (8 of 12) of the HLA-B7 volunteers induced a CD8⁺-T-cell response to at least one of the peptides. Two of these peptides, Nef 135-143 and Gag 267-277, elicited a positive response in four and five volunteers, respectively. Finally, there was an anti-HIV-specific response in three of four (75%) vaccinated volunteers presenting the HLA-B8 molecule. Three of the four HLA-B8 volunteers tested responded to the Gag 259-267 peptide.

DISCUSSION

We describe here the complete immunology data of the induced, booster, and sustained T-cell responses to vaccination with HIV-lipopeptides in seronegative volunteers. Table 6

TABLE 5. Positive MHC class I-restricted CD8⁺-T-cell response to HIV-1 peptides

HLA type	Frequency ^a of responding subjects	No. of CD8 ⁺ HIV-1 peptides tested	Epitopes recognized in IFN- γ ELISPOT assay	
			Name	Sequence
A1	2/5	3	Nef 121-128	FPDWQNYT
			Nef 195-202	ARELHPEY
A2	6/8	15	Nef 82-91	KAA(L/V)DLSHFL
			Nef 83-91	AAVDLSHFL
			Nef 84-91	AVDLSHFL
			Nef 136-145	PLTFGWYCYKL
			Nef 137-145	LTFGWYCYKL
			Nef 190-198 (2)	ALHHVAREL
			Gag 260-268	EIYKRWII
			Gag 267-274	ILGLNKIV
			Gag 275-283	RMYSPTSIL
			Nef 84-92	AVDLSHFLK
			Gag 266-275	IILGLNKIVR
			Gag 267-275	ILGLNKIVR
			Nef 80-87	TYKAAVDL
A3/A11	4/8	10	Nef 134-143	RYPLTFGWYCY
			Gag 261-270	IYKRWII
A24	4/4	8	Gag 276-283	MYSPTSIL
			Nef 68-76	FPVTPQVPL
#B7	8/12	13	Nef 71-79	TPQVPLRPM
			(R/T)PQVPLRPM	
			Nef 71-81	TPGPGVRY
			Nef 128-135	TPGPGVRYPL
			Nef 128-137	TPGPGVRYPL
			Nef 135-143	YPLTFGWYCY
			Gag 254-262	PPIPVGIEY
			Gag 267-277	I(Y/L)GLNKIVRMV
			Nef 182-189	EWRFDSSL
			Gag 259-267	GEIYKRWII
B8	3/4	3		

^a That is, the ratio of the number of vaccinated volunteers recognizing at least one peptide versus the number of volunteers bearing the HLA restriction molecule who have been tested with the same peptides.

^b That is, HIV-1 peptides eliciting specific CD8⁺-T-cell responses after HIV-1 vaccination. Peptides frequently recognized by T cells are in boldface in columns 4 and 5.

summarizes the percentage of positive responders after three (W20) and four (W52) injections and the sustained immune response (at W104) in the 28 volunteers enrolled in the present study. Detailed analyses of the antibody responses obtained from the vaccinated volunteers are not presented here, but a summary of the specific immunoglobulin G (IgG) induced is given in Table 6. After three injections, IgG and CD4⁺ and CD8⁺ T cells (as determined by IFN- γ ELISPOT assay) were detected in 89, 79, and 71% of the vaccinated volunteers, respectively. After the fourth injection, we observed an increase in the number of positive responders, but this increase was not significant ($P > 0.05$ compared to three injections), even if >85% of the volunteers had a positive response to at least one of the immunological parameters tested. At this time, >50% of them presented a multispecific immune response. At W104, a sustained immune response was observed in >50% of the volunteers. We also compare the results obtained by standard chrome release assay (data not shown) and ELISPOT method for the measure of the CD8⁺-T-cell response. The lower number of positive responders obtained by chrome release assay could in part be explained by the fact that we started the CD8⁺-T-cell analysis by using HIV-1 long peptides derived from the vaccine and not the optimal HIV-1 CD8⁺-T-cell epitopes. Processing of long peptides in vitro cannot be controlled, and this is not necessarily the best way to obtain CD8⁺ T cells. These results also showed that combining the

TABLE 6. Summary of antibody, proliferative, and CD8⁺-T-cell induction results

Variable and wk	No. of volunteers				
	R (%)	Mono-R ^d	Poly-R ^e	-QS21-R ^f	+QS21-R ^g
Antibody^a					
W20	25/28 (89)	3	22	11/14	14/14
W52	26/28 (93)	5	21	12/14	14/14
W104	16/28 (57)	8	8	4/14	12/14
Proliferation^b					
W20	19/24 (79)	5	14	9/11	10/13
W52	24/26 (92)	6	18	11/13	13/13
W104	14/21 (67)	6	8	3/10	11/11
CD8⁺ response^c					
(IFN- γ)					
W20	12/17 (71)	5	7	8/9	4/8
W52	19/22 (86)	6	13	10/11	9/11
W104	13/23 (57)	5	8	7/11	6/12

^a The percentage of positive responders for antibody induction after immunization at W20, W52, and W104 is given in parentheses in col. 2. The ratio indicates the number of positive responders among the volunteers tested.

^b The percentage of positive responders for the induction of a specific HIV-1 proliferative response is given in parentheses in col. 2.

^c The percentage of CD8⁺-T-cell responses obtained after immunization is given in parentheses in col. 2.

^d That is, the number of volunteers in whom an antibody, proliferative, or CD8⁺-T-cell monospecific response (Mono-R) was induced.

^e That is, the number of volunteers in whom an antibody, proliferative, or CD8⁺-T-cell polyspecific response (Poly-R) was induced (Poly-R is defined as the recognition of at least two peptides).

^f Ratio of the positive responders to the number of volunteers immunized with lipopeptide alone (-QS21-R).

^g Ratio of positive responders to the number of volunteers immunized with lipopeptide and QS21 adjuvant (+QS21-R).

ELISPOT method and a bank of optimum CD8⁺-T-cell epitopes was a better approach to characterize this population.

In the present study we also attempted to measure the effect of the QS21 adjuvant on the kinetics of the induced immune response after multiple immunizations because this adjuvant had been shown to elicit CTL responses in animal models (1). Table 6 presents the effect of QS21 adjuvant on the immune response induced in 28 volunteers. After three injections (W20), 100% of the volunteers immunized with adjuvant and 78.6% of the volunteers immunized without adjuvant showed induced antibody responses, but the percent difference was not significant ($P > 0.05$). After the fourth injection (W52), 100% of the volunteers immunized with adjuvant presented both antibody and proliferative responses, but the QS21 effect was not significant ($P > 0.05$ compared to the group immunized without adjuvant). In contrast, a significant difference was found when the long-term responses of B and CD4⁺ T cells were evaluated. Thus, a QS21 positive effect was observed on the sustained B-cell responses ($P = 0.01$) and CD4⁺-T-cell responses ($P = 0.007$) compared to the group immunized without adjuvant.

In a mouse study, anti-HIV antibodies were effectively induced by the lipopeptide construct (34). However, the authors of that study provided evidence that the biological activity of these antibodies, i.e., their ability to neutralize HIV-1 infectivity in vitro, was influenced by the presence of adjuvant. This result could be a marker of antibody maturation. The secretion pattern of cytokine influences isotype switch of immunoglob-

ulin. In this regard, distribution of anti-HIV immunoglobulin isotype after lipopeptide vaccination has been tested in some volunteers (data not shown). At W20, antibodies elicited by three immunizations were found to belong predominantly to the IgG1 subclass. The fourth immunization produced a more complex isotype distribution, and we were able to detect IgG1, IgG2, IgG3, IgG4, and IgA in the sera from the vaccinated volunteers, a finding that indicates the production of cytokines from Th1 and Th2 cells. At W104, a sustained CD8⁺-T-cell response was observed in 64% of the positive responders immunized with lipopeptide alone and in 50% of the positive responders immunized with adjuvant, but the difference was not significant ($P > 0.05$). Therefore, we cannot reach a definite conclusion on the effect of this adjuvant on the CD8⁺-T-cell response in vaccination.

We showed here that the majority of positive responders induced and maintained multiepitopic CD8⁺-T-cell responses. This is a major finding, since the monoepitopic CTL response induced in a preclinical SIV-macaque model after lipopeptide immunization was found to select virus escape mutants (27). This finding is in agreement with observations in humans, where the transfer of an HIV-1-specific CTL clone to an AIDS patient led to the emergence of HIV variants and subsequent disease progression (20).

To investigate the CD8⁺-T-cell response directed against a large variety of HIV-1 peptides (59 different HIV-1 peptides), we performed an IFN- γ ELISPOT assay (after a one-step stimulation) in 23 of the 28 vaccinated volunteers. We showed that 31 of 59 HIV-1 CD8⁺-T-cell epitopes tested induced stimulation after vaccination. For the most common HLA molecules, the frequency of T-cell recognition was higher for Nef 136-145, Nef 137-145, Gag 260-268, and Gag 267-274 (presented by HLA-A2); Nef 84-92 (HLA-A11); Gag 276-283 (HLA-A24), Nef 68-76, Nef 71-79, Nef 135-143, and Gag 267-277 (HLA-B7 superfamily); and Gag 259-267 (HLA-B8). The proteasome is one of the major enzymatic systems involved in the cellular processing that results in the release of peptides. Interestingly, our lipopeptide construct, Nef 117-147, contains all of those CD8⁺-T-cell epitopes described by Lucchiari-Hartz et al. that were generated by 20S proteasome (24). We tested the five HIV-1 peptides described, and we showed that all five of these peptides elicited CD8⁺-T-cell responses in vaccinated volunteers. Similarly, we used the same approach to study the digestion of a long peptide 66-100 that includes the sequence of our lipopeptide Nef 66-97 (8). We identified five HIV-1 CD8⁺-T-cell epitopes—Nef 68-76, Nef 71-79, and Nef 71-81 (presented by HLA-B7 superfamily), Nef 83-91 (HLA-A2), and Nef 84-92 (HLA-A3/A11)—that were naturally produced in humans after vaccination. Similarly, with the Gag 253-284 lipopeptide, 11 HIV-1 peptides elicited CD8⁺-T-cell responses, a finding which suggests that this region also could be efficiently cleaved by the 20S proteasome. In fact, our results showed that the lipopeptide vaccine contains CD8⁺-T-cell epitopes that can be specifically induced after vaccination and that the elicited CD8⁺ T-cell responses are not necessarily randomly distributed among the different subjects but may depend on the HLA class I distribution and precise molecular peptide processing. Finally, the cartography presenting the HIV-1 CD8⁺-T-cell epitopes specifically induced after vaccination in healthy volunteers could be helpful in the future to

better understand the immunological role on the viral protection.

In a previous report, Dalod et al. (11) showed by using IFN- γ ELISPOT assay and a bank of HIV-1 CD8⁺-T-cell epitopes that anti-HIV CD8⁺-T-cell responses in primary and chronic infection were different. Interestingly, Nef 136-145 (HLA-A2) was frequently recognized by both chronically infected patients and vaccinated volunteers, whereas the peptide was not or seldom recognized by subjects with primary HIV infection. In contrast, we showed that peptide Nef 71-81 was recognized by few HLA-B7-typed volunteers and that peptides Nef 73-82 (HLA-A3/A11) and Nef 134-141 (HLA-B27) were not recognized by T cells from vaccinated volunteers, whereas these peptides were frequently recognized at both stages of HIV infection. Finally, we showed that some peptides, i.e., Nef 84-92 (HLA-A11), Nef 135-143 (HLA-B7 superfamily), and Gag 259-267 (HLA-B8), were recognized after vaccination, as observed by Dalod et al. in patients at both stages of HIV infection. These data suggest that T-cell epitopes induced by vaccination volunteers could be different from those observed in HIV-infected patients. However, this question could be answered only through a detailed immunological analysis of a cohort of HIV-infected patients compared to vaccinated volunteers.

On the other hand, we have previously reported that some mutations in the CD8⁺ T cell could lead to a reduction in CTL activity and that this kind of mutation could facilitate the selection of HIV variants that can avoid the cellular immune response. We showed, notably in Nef CD8⁺-T-cell epitopes, that mutations in the major anchor positions prevented binding and thus impaired recognition of the HLA-peptide complex by the TCR (10). Mutations in the major TCR positions could have the same consequence and prevent the recognition of the complex by specific CD8⁺ T cells. However, the avidity for a given peptide of an induced T cell could be modified by antigen restimulation, and this could result in an expansion of this specific T cell. If the TCR avidity for a wild-type peptide is very high, this could result in the recognition of a mutant peptide under certain circumstances. Therefore, we tested the possibility that repeated injections of lipopeptide vaccine could generate CD8⁺ T cells able to recognize HIV-1 wild-type peptide and HIV-1 mutated peptides by cross-reaction. We selected two peptides described with one amino acid substitution: Gag 267-277 and Nef 82-91. We showed that in one volunteer the wild-type Gag 267-277 peptide could elicit a response after three injections and that both the wild type and the mutant elicit a response after four injections. The same result was obtained for the Nef 82-91 peptide with another volunteer since the wild-type Nef 82-91 peptide elicited a response at W20 (after three injections), and the mutant elicited a specific response only after the boost (four injections). These results are in agreement with the hypothesis that increased avidity of the TCR for a given peptide can explain the cross-reactivity observed for a modified peptide. However, these results concern only two peptides, and they do not allow us to conclude that the vaccine will protect against variant viruses circulating in the population.

CD4⁺ T cells are necessary for induction and maintenance of the effector functions of CD8⁺ T cells. First, CD4⁺ T cells engage and condition the professional antigen-presenting cells,

e.g., dendritic cells. These conditioned antigen-presenting cells then become able to stimulate CD8⁺ T cells (21, 30). A study in which humans were immunized with a lipopeptide hepatitis B virus vaccine showed that the CD4⁺-T-cell response is important for the development of CTL responses (23). We also found that T helper activity was required for the induction of specific CD8⁺-T-cell responses. The HIV-specific proliferative CD4⁺-T-cell response of PBMCs from vaccinated volunteers was associated with the induction of HIV-specific CTL activity, but 1 of the 28 volunteers (volunteer V4.30) did not present this association, although a long-lasting multi-epitopic CD8⁺-T-cell response was present.

The lipopeptide vaccine contains immunodominant HIV-1 CD4⁺ epitopes. We showed that 85% (23 of 27) of the volunteers gave a proliferative response to the long peptide G2 at W20 or W52. Wilson et al. recently identified 11 CD4⁺-conserved HIV-1-derived epitopes, 3 in Gag and 8 in Pol proteins, that are capable of binding a minimum of 7 HLA-DR types (37). In fact, two of the Gag peptides are nested within epitopes reported by Rosenberg et al. and found to be associated with the control of viremia (31). A strong association existed between recognition of the parental recombinant HIV-1 protein and the corresponding CD4⁺ peptides, which suggests that these peptides are the epitopes that are processed and presented during the course of HIV-1 infection. Moreover, two of the three highly cross-reactive binding peptides identified in p24 Gag protein, the Gag 259-273 (GEIYKR WIILGLNKI) and Gag 263-277 (KRWIILGLNKIVRMY) peptides, are present in our G2 lipopeptide (Gag 253-284). These two peptides would be expected to bind in at least 89% and up to 95% of the human population. These results could easily explain the very high frequency of positive responders detected in our study. Identification of highly conserved, widely recognized epitopes is one of the critical steps in developing such vaccines. These two Gag CD4⁺-T-cell epitopes are conserved in >94% of clade B that has been analyzed. These highly cross-reactive HLA-DR binding peptides are well conserved in a variety of HIV-1 isolates, 58% for Gag 259-273 and 85% for Gag 263-277, in all of the HIV-1 isolates examined.

Our results indicated that CD4⁺- and CD8⁺-T-cell epitopes included in the HIV-1 lipopeptide are efficiently processed in humans. These lipopeptides have the potential to be generated in many different epitopes that can react with different major histocompatibility complexes (MHCs), which make it possible to use this approach in individual patients, regardless of their MHC. Moreover, the sustained multi-epitopic HIV-1 CD4⁺- and CD8⁺-T-cell responses obtained in our clinical trial might have important implications also for immunotherapy and for understanding the role of these HIV-1 T cells in the control of infection. In order to improve CD4⁺- and CD8⁺-T-cell responses obtained by lipopeptide vaccination, new formulations will be tested in phase I and II clinical trials. Finally, to avoid induction of an immune response against vector vaccines, lipopeptides can be used also as a boost after immunization with recombinant poxvirus, DNA, or MVA containing a complex combination of HIV-genes.

ACKNOWLEDGMENTS

We thank Christelle Troadec and Bénédicte Charmeteau for outstanding technical assistance. We also thank the volunteers for their

cooperation and the ANRS for continuous support and assistance in the recruitment and selection of volunteers. We acknowledge the assistance of Marie-Pierre Treilhou and Naïma Kerbouche from Rothschild Hospital vaccine trial center and Sandra Fournier, Vincent Feuille, and Hubert Poncelet from Pasteur Hospital.

This study was supported by grants from Institut National de la Santé et de la Recherche Médicale (INSERM) and ANRS.

REFERENCES

- Ahlers, J. D., N. Dunlop, C. D. Pendleton, M. Newman, P. L. Nara, and J. A. Berzofsky. 1996. Candidate HIV type 1 multideterminant cluster peptide-P18MN vaccine constructs elicit type 1 helper T cells, cytotoxic T cells, and neutralizing antibody, all using the same adjuvant immunization. *AIDS Res. Hum. Retrovir.* 12:259-272.
- Allen, T. M., T. U. Vogel, D. H. Fuller, B. R. Mothe, S. Steffen, J. E. Boyson, T. Shipley, J. Fuller, T. Hanke, A. Sette, J. D. Altman, B. Moss, A. J. McMichael, and D. I. Watkins. 2000. Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *J. Immunol.* 164:4968-4978.
- Andrieu, M., E. Loing, J. F. Desoutter, F. Connan, J. Choppin, H. Gras-Masse, D. Hanau, A. Dautry-Varsat, J. G. Guillet, and A. Hosmalin. 2000. Endocytosis of an HIV-derived lipopeptide into human dendritic cells followed by class I-restricted CD8⁺ T lymphocyte activation. *Eur. J. Immunol.* 30:3256-3265.
- Appay, V., P. R. Dunbar, M. Callan, P. Klennerman, G. M. A. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael, and S. L. Rowland-Jones. 2002. Memory CD8⁺ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* 8:379-385.
- Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael, and S. L. Rowland-Jones. 2000. HIV-specific CD8⁺ T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* 192:63-75.
- BenMohamed, L., H. Gras-Masse, A. Tartar, P. Daubersies, K. Brahimi, M. Bossus, A. Thomas, and P. Druilhe. 1997. Lipopeptide immunization without adjuvant induces potent and long-lasting B, T helper, and cytotoxic T lymphocyte responses against a malaria liver stage antigen in mice and chimpanzees. *Eur. J. Immunol.* 27:1242-1253.
- Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, R. Forster, S. Rowland-Jones, R. P. Sekaly, A. J. McMichael, and G. Pantaleo. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 410:106-111.
- Choppin, J., W. Cohen, A. Bianco, J. P. Briand, F. Connan, M. Dalod, and J. G. Guillet. 2001. Characteristics of HIV-1 Nef regions containing multiple CD8⁺ T-cell epitopes: wealth of HLA-binding motifs and sensitivity to proteasome degradation. *J. Immunol.* 166:6164-6169.
- Cohen, J. 2001. Debate begins over new vaccine trials. *Science* 293:1973.
- Coullin, I., B. Culmann-Penciolelli, E. Gomard, J. Choppin, J. P. Levy, J. G. Guillet, and S. Saragosti. 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein. *J. Exp. Med.* 180:1129-1134.
- Dalod, M., M. Dupuis, J. C. Deschemin, C. Goujard, C. Deveau, L. Meyer, N. Ngo, C. Rouzioux, J. G. Guillet, J. F. Delfraissy, M. Sinet, and A. Venet. 1999. Weak anti-HIV CD8⁺ T-cell effector activity in HIV primary infection. *J. Clin. Invest.* 104:1431-1439.
- Dalod, M., M. Dupuis, J. C. Deschemin, D. Sicard, D. Salmon, J. F. Delfraissy, A. Venet, M. Sinet, and J. G. Guillet. 1999. Broad intense anti-human immunodeficiency virus (HIV) ex-vivo CD8⁺ responses in HIV type-1-infected patients: comparison with anti-Epstein-Barr virus responses and changes during antiretroviral therapy. *J. Virol.* 73:7108-7116.
- Esparza, J., and N. Bhamarapravati. 2000. Accelerating the development and future availability of HIV-1 vaccines: why, when, where, and now? *Lancet* 355:2061-2066.
- Gahery-Segard, H., G. Pialoux, B. Charmeteau, S. Sermet, H. Poncelet, M. Raux, A. Tartar, J. P. Levy, H. Gras-Masse, and J. G. Guillet. 2000. Multi-epitopic B- and T-cell responses induced in humans by a human immunodeficiency virus type 1 lipopeptide vaccine. *J. Virol.* 74:1694-1703.
- Hanke, T., and A. J. McMichael. 2000. Design and construction of an experimental HIV-1 vaccine for a year-2000 clinical trial in Kenya. *Nat. Med.* 6:951-955.
- Hanke, T., R. V. Samuel, T. J. Blanchard, V. C. Neumann, T. M. Allen, J. E. Boyson, S. A. Sharpe, N. Cook, G. L. Smith, D. I. Watkins, M. P. Cranage, and A. J. McMichael. 1999. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multi-epitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J. Virol.* 73:7524-7532.
- Kalams, S. A., and B. D. Walker. 1998. The critical need for CD4 help in

- maintaining effective cytotoxic T lymphocyte responses. *J. Exp. Med.* **188**: 2199–2204.
18. Kaul, R., T. Dong, F. A. Plummer, J. Kimani, T. Rostron, P. Kiama, E. Njagi, E. Irungu, B. Farah, J. Oyugi, R. Chakraborty, K. S. MacDonald, J. J. Bwayo, A. McMichael, and S. L. Rowland-Jones. 2001. CD8⁺ lymphocytes respond to different HIV epitopes in seronegative and infected subjects. *J. Clin. Invest.* **107**:1303–1310.
 19. Klinguer, C., D. David, M. Kouach, J. M. Wieruszeski, A. Tartar, D. Marzin, J. P. Levy, and H. Gras-Masse. 1999. Characterization of a multi-lipo peptide mixture used as an HIV-1 vaccine candidate. *Vaccine* **18**:259–267.
 20. Koenig, S., A. J. Conley, Y. A. Brewah, G. M. Jones, S. Leath, L. J. Boots, V. Davey, G. Pantaleo, J. F. Demarest, and C. Carter. 1995. Transfer of HIV-1 specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nat. Med.* **1**:330–336.
 21. Lanzavecchia, A. 1998. Immunology licence to kill. *Nature* **393**:413–414.
 22. Levy, J. P. 1998. AIDS vaccine development. *Science* **280**:806–807.
 23. Livingston, B. D., C. Crimi, H. Grey, G. Ishioka, F. V. Chisari, J. Fikes, H. Grey, R. W. Chesnut, and A. Sette. 1997. The hepatitis B virus-specific CTL responses induced in humans by lipopeptide vaccination are comparable to those elicited by acute viral infection. *J. Immunol.* **159**:1383–1392.
 24. Lucchiari-Hartz, M., P. M. Van Endert, G. Lauvau, R. Maier, A. Meyerhans, D. Mann, K. Eichmann, and G. Nierdermann. 2000. Cytotoxic T lymphocytes epitopes of HIV-1 nef: generation of multiple definitive major histocompatibility complex class I ligands by proteasomes. *J. Exp. Med.* **191**:239–252.
 25. McMichael, A. J., and S. L. Rowland-Jones. 2001. Cellular immune responses to HIV. *Nature* **410**:980–987.
 26. Mortara, L., H. Gras-Masse, C. Rommens, A. Venet, J. G. Guillet, and I. Bourgault-Villada. 1999. Type 1 CD4⁺ T-cell help is required for induction of antipeptide multispecific cytotoxic T lymphocytes by a lipopeptidic vaccine in rhesus macaques. *J. Virol.* **73**:4447–4451.
 27. Mortara, L., F. Letourneur, H. Gras-Masse, A. Venet, J. G. Guillet, and I. Bourgault-Villada. 1998. Selection of virus variants and emergence of virus escape mutants after immunization with epitope vaccine. *J. Virol.* **72**:1403–1410.
 28. Pialoux, G., H. Gahery-Segard, S. Sermet, H. Poncelet, S. Fournier, L. Gerard, A. Tartar, H. Gras-Masse, J. P. Levy, J. G. Guillet, and A. V. S. Team. 2001. Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers. *AIDS* **15**:1239–1249.
 29. Pitcher, C. J., C. Quitner, D. M. Peterson, M. Connors, A. Koup, V. C. Maino, and L. J. Picker. 1999. HIV-1-specific CD4⁺ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat. Med.* **5**:518–525.
 30. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and a T-killer cell. *Nature* **393**:474–478.
 31. Rosenberg, E., J. Billingsley, A. Caliendo, S. Boswell, P. Sax, S. Kalans, and B. Walker. 1997. Vigorous HIV-1 specific CD4⁺ T-cell responses associated with control of viremia. *Science* **278**:1447–1450.
 32. Russell, N. D., M. G. Hudgens, R. Ha, C. Havenar-Daughton, and M. J. McElrath. 2003. Moving to human immunodeficiency virus type 1 vaccine efficacy trials: defining T-cell responses as potential correlates of immunity. *J. Infect. Dis.* **187**:226–242.
 33. Sauzet, J. P., B. Deprez, F. Martinon, J. G. Guillet, H. Gras-Masse, and E. Gomard. 1995. Long-lasting anti-viral cytotoxic T lymphocytes induced in vivo with chimeric-multirestricted lipopeptides. *Vaccine* **13**:1339–1345.
 34. Sauzet, J. P., C. Moog, A. Krivine, F. Martinon, M. Bossus, H. Gras-Masse, A. Tartar, J. G. Guillet, and E. Gomard. 1998. Adjuvant is required when using Env lipopeptide construct to induce HIV type 1-specific neutralizing antibody responses in mice in vivo. *AIDS Res. Hum. Retrovir.* **14**:901–909.
 35. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, K. T. Racz, M. Dalesandro, B. J. Scallan, J. Gharyed, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8⁺ lymphocytes. *Science* **283**:857–860.
 36. Vitiello, A., G. Ishioka, H. M. Grey, R. Rose, P. Farness, R. LaFond, L. Yuan, F. V. Chisari, J. Furze, R. Bartholomeuz, and R. Chesnut. 1995. Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. *J. Clin. Invest.* **95**:341–349.
 37. Wilson, C. C., B. Palmer, S. Southwood, J. Sidney, Y. Higashimoto, E. Appella, R. Chesnut, A. Sette, and B. D. Livingston. 2001. Identification and antigenicity of broadly cross-reactive and conserved human immunodeficiency virus type 1-derived helper T-lymphocyte epitopes. *J. Virol.* **75**:4195–4207.