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Induction of Broad Cross-Subtype-Specific HIV-1 Immune Responses by a Novel Multivalent HIV-1 Peptide Vaccine in Cynomolgus Macaques

Ali Azizi,*† David E. Anderson,*† José V. Torres,‡ Andrei Ogrel,* Masoud Ghorbani,* Catalina Soare,*§ Paul Sandstrom,§ Jocelyne Fournier,§ and Francisco Diaz-Mitoma1*†

One of the major obstacles in the design of an effective vaccine against HIV-1 is its antigenic variation, which results in viral escape from the immune system. Through a bioinformatics approach, we developed an innovative multivalent HIV-1 vaccine comprised of a pool of 176 lipidated and nonlipidated peptides representing variable regions of Env and Gag proteins. The potency and breadth of the candidate vaccine against a panel of HIV-1 subtypes was evaluated in nonhuman primate (cynomolgus macaques) and humanized mouse (HLA-A2.1) models. The results demonstrate strong immunogenicity with both breadth (humoral and cellular immunity) and depth (immune recognition of widely divergent viral sequences) against heterologous HIV-1 subtypes A–F. The Journal of Immunology, 2008, 180: 2174–2186.

Antigenic variation has thwarted HIV vaccine development for over two decades (1, 2). By design, the majority of the current vaccine strategies target specific epitopes of one HIV strain; however, it is important for a vaccine to have the ability to induce an immune response against a range of HIV variants.

The role of neutralizing Abs (Nabs)2 in controlling HIV-1 infection is undeniable, but to date, only a limited number of Abs with neutralization capabilities against primary isolates has been identified (3–6). The crystallographic structure of gp120 indicates that the protein is covered by carbohydrates which facilitate viral escape from Nabs (7, 8). The genetic variability in HIV-gp120 between groups M, N, and O also affects the induction of Nabs and allows the virus to escape immune recognition triggered by humoral responses directed against gp120 epitopes (9, 10). The challenge associated with inducing Nabs against primary isolates in light of the efficacy of T cells in reducing viral loads in HIV-1-infected individuals has encouraged focus on cell-mediated immune responses (11).

In this study, we report on the development of a novel HIV-1 peptide vaccine based on combined prediction algorithms, HLA polymorphism in human loci, and molecular modeling. More than 200 HIV-1 genome sequences were chosen from the HIV Los Alamos sequence database and their epitopes were analyzed. We synthesized many variants of several epitopes, representing the variable regions of Env and Gag that were either lipidated or nonlipidated. The presence of lipidated peptide variants served both to elicit a CTL response and to activate innate immunity due to TLR2 recognition of the lipid moiety present on the peptides (12–14). To evaluate the relative merits of immunization with lipidated, nonlipidated, or both forms of our peptides, in terms of inducing cellular immunity, groups of HLA-A2.1 mice were immunized with lipidated and nonlipidated peptide variants alone or in combination. As controls, mice were either immunized with adjuvant alone or left unvaccinated entirely. The group of mice that received a mixture of 5 lipidated and 7 lipidated peptide mixtures, comprising a total of 176 unique peptide variants, showed a higher level of specific antiviral CD8+ T cell responses to heterologous HIV-1 subtypes A–D than did mice immunized with only lipidated or nonlipidated peptides. Based on these results obtained in mice, six macaques were immunized with the mixture of lipidated and nonlipidated peptides. The breadth of cellular and humoral immunity induced in vaccinated macaques was measured against heterologous HIV-1 subtypes. Results from this study demonstrated that the multivalent HIV-1 candidate vaccine was able to generate a broad cell-mediated immune response against a wide range of heterologous HIV-1 subtypes A–F. Immunophenotypic characteristics of specific T cells in immunized macaques indicated an up-regulation in memory CD4+ and CD8+ T cell subsets. The candidate vaccine was also able to induce a relatively high level of IgG and IgA Ab binding and moderate neutralizing activity against T cell line-adapted (TCLA) and primary HIV-1 isolates. To investigate whether the immune response induced after vaccination with the HIV-1 peptides could lead to protective immunity, vaccinated HLA-A2.1 mice were challenged and a significant reduction in viral titers was revealed. These findings indicate that the selected multivalent peptides in the vaccine are able to induce broad immunogenicity against various worldwide HIV-1 isolates.

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2Abbreviations used in this paper: Nab, neutralizing Ab; DP, double positive; TCLA, T cell line adapted; TFA, triluoroacetic acid; rVV, recombinant vaccinia virus; ICS, intracellular cytokine staining; FCS, forward scatter; SSC, side scatter; SI, stimulation index; SFC, spot-forming cell; EM, effector memory; CM, central memory; SP, single positive; RLU, relative luminescence unit; SHIV, simian HIV; aa, amino acid.

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Sequences of Vaccine Peptides

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*Variable peptides are indicated in red with inserted amino acid below original sequence.*
Materials and Methods

Vaccine design, synthesis, isolation, and analysis

We examined 21 subtype A, 128 subtype B, 51 subtype C, 17 subtype D, and 33 subtype E sequences (Los Alamos Database) in the formulation of our vaccine. Vaccine formulations are based on five variable epitopes in HIV-1 Env and two variable epitopes in HIV-1 Gag (see Fig. 1A). All peptides were synthesized by automated solid-phase F-moc chemistry on a Pioneer Peptide Synthesizer (Applied Biosystems). All protected amino acid (aa) and resins were purchased from Novabiochem (EMD Biosciences). Reagents and palmitic acid were obtained from Sigma-Aldrich. All solvents were obtained from Omnisolv (EMD Chemicals).

Briefly, peptides were assembled by stepwise approach using a four times excess of amino acids on a Rink-Amide Novabiochem resin. Equimolar amount of amino acids were added at multiple positions with variability within an epitope. For purposes of lipidation, a portion of each of gp120-derived peptide formulations, and the entire amount of Gag-derived peptides were elongated by tripeptide linker, Lys-Ser-Ser. N-terminal Lys was double lipitated at its two free amino groups using a 10-fold excess of palmitic acid for 12 h. Following completion of peptide synthesis, peptides were cleaved from the solid-phase support and the protective groups were removed with trifluoroacetic acid (TFA) containing “mixture” (TFA/water/ phenol/thioanisole/triisopropylsilane (83:5:5:5:2)) for 3 h. Crude peptides were precipitated in cold ether, centrifuged at 1000 g for 5 min, and the ether was removed. The extraction procedure was repeated three times, and samples were finally dried under nitrogen gas. Nonlipidated peptide mixtures were eluted using H2O/CH3CN solvent system on reverse-phase C18 column (Vydac), and all lipidated peptide formulations were collected by size-exclusion chromatography on Sephadex LH-20 in DMSO. Organic solvents were reduced to a minimum, and the peptides were then resuspended in water and finally lyophilized.

Analytical reverse-phase-HPLC was conducted using a Vydac C18 column (4.6 × 300 mm; Mandel Scientific) at 214 nm. Peptides were eluted with a 0–100% gradient over 60 min at flow rate of 1 ml/min using 0.1% TFA in H2O and 0.1% TFA in CH3CN as solvents. All peptides were characterized by MALDI-TOF mass spectrometry using a Voyager-DE Biospectrometry Workstation (Applied Biosystems), equipped with delayed ion extraction. All expected peptide masses were identified on mass spectra.

Animal studies and immunization

Twelve cynomolagus macaques serologically negative for SIV were maintained in accordance with the institutional animal care protocols (ACC 03025) of Health Canada. Fifty micrograms of each of the five nonlipidated (80 variants) and seven lipidated (96 variants) formulations were resuspended in sterile saline and mixed at a 1:1 ratio with Montanide ISA-51 adjuvant. The vaccine was injected into six animals (nos. 079, 012, 059, 020, 011, and 013) s.c. 2 cm from the inguinal area in the anterior aspect of the thigh, close to the draining inguinal lymph nodes. Animals were vaccinated at months 0, 1, 3, 7, and 10. Another six animals were left unvaccinated because in a previous experiment, no adaptive immune response difference between unvaccinated and montanide alone group was observed (15).

Six to ten nanomolar female C57BL/6-TgN (HLA-A2.1) mice which contain the full human HLA-A2.1 gene were purchased from The Jackson Laboratory and housed in the animal facility at University of Ottawa in accordance with the Children’s Hospital of Eastern Ontario-68-approved protocol. Four groups (n = 4) of mice (adjuvant alone, lipidated peptides, nonlipidated peptides, and a mixture of peptides) were immunized four times s.c. at the base of the tail. Each mouse received a total of 120 μg of immunogen (1:1 ratio of Montanide ISA-51 adjuvant to peptides in PBS) per vaccination. Ten days after the last immunization, the mice were euthanized and cell-mediated immune response was measured.

Recombinant vaccinia viruses (rVV), recombinant proteins, and peptides

The following recombinant vaccinia viruses (vP1286, vT173, vT176, vBD3, vT198R, vT234, vABT 408, vT331, vABT489, and vT1170) were obtained from National Institutes of Health AIDS Research and Reference Reagent Program and used in intracellular cytokine staining (ICS) and ELISPOT assays. vP1286 expresses the MN (subtype B) Env, vT173 expresses the 94UG114.1 (subtype D) Env, vT176 expresses the 90CF402.1 (subtype E) Env, vBD3 expresses the 89.6 (subtype B) Env, vT198R expresses the 92UG037.1 (subtype A) Env, vT234 expresses the 93BR02 (subtype B) Env, vABT 408 expresses the 92UG037.1 (subtype B) Env, vT331 expresses the 96ZM651.8 (subtype C) Env/Vag/Gag/Pol, and vABT489 expresses the IIBB (subtype B) Env/Vag/Gag/Pol, and empty vT1170 as a control.

The recombinant proteins HIV-1 IIB gp160 (subtype B) and HIV-1 MN gp120 (subtype B) were purchased from Advanced Biotecnologies. The recombinant protein HIV-1 CM gp120 (subtype E) was purchased from Protein Sciences. The recombinant HIV-1 gp120 proteins subtype C (96ZM651), HIV-1 gp120 subtypes A/E (93TH975), HIV-1gp120 subtype B (SF162), and HIV-1 peptides corresponding to subtypes A–D were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

Proliferation assays

PBMCs from animals were resuspended at 2 × 10^6 cells/ml in RPMI 1640 containing 10% FCS, 50 μM 2-ME, and 100 μM penicillin/streptomycin. A 100-μl aliquot containing 2 × 10^5 cells was added to each well of a 96-well plate. The recombinant proteins HIV-1 IIB gp160 (subtype B), HIV-1 MN gp120 (subtype B), HIV-I CM gp120 (subtype B), HIV-1 96ZM651 gp120 (subtype C), HIV-1 93TH975 gp120 (subtypes A/E), and HIV-1 SF162 gp120 (subtype B) were added (1 μg/ml) to each well in triplicate. Cells were also stimulated with pooled HIV-1 Env peptides. As a positive control, cells were also stimulated with PMA and ionomycin (Sigma-Aldrich). After 5 days of culture, 1 μCi [3H]thymidine (Amer sham) was added to each well. Following 16 h of incubation, cells were harvested onto glass-fiber filter mats and thymidine incorporation was measured with a Microbeta beta counter (Wallac). Results were expressed as stimulation index calculated by dividing the mean cpm of cells incubated with HIV-1 proteins by the cpm of cells incubated with medium alone.

ELISPOT assay

PBMCs were stimulated with 2 PFU of rVVs expressing HIV-1 genes from subtypes A–F or empty rVV as a control (National Institutes of Health AIDS Research and Reference Reagent Program) for 90 min. After washing, 2.5 × 10^5 cells were added to plates coated with anti-mouse IFN-γ (Mabtech). After 40 h at 37°C, cells were removed, washed with PBS plus 0.05% Tween 20, and incubated with 1 μg/ml biotinylated anti-mouse IFN-γ (Mabtech) for 2 h at room temperature. After washing, streptavidin-ALP-PQ (Mabtech) in PBS plus 0.5% FCS was added and incubated for 1 h at room temperature. The plates were washed as above and developed with 100 μl/well 5-bromo-4-chloro-3-indolyl phosphate/NBT alkaline phosphatase (Moss) for 20 min at room temperature. The reaction was stopped by rinsing the plates with tap water. The numbers of spots were counted and expressed as the mean count ± SD. Anti-mouse IFN-γ Ab (mAb AN18; Mabtech) and biotinylated anti-mouse IFN-γ Ab (mAb R4–6A2–bio; Mabtech) were used in mice ELISPOT assay.

Intracellular cytokine staining

PBMCs from vaccinated and unvaccinated macaques were stimulated with 2 PFU of rVVs expressing HIV-1 genes from subtypes A–F or empty rVV as a control (National Institutes of Health AIDS Research and Reference Reagent Program). Cells were washed after 90 min and suspended in RPMI 10 and incubated for 16–18 h in monensin (Golgistop; BD Biosciences). Sixteen hours after incubation, the cells were washed with 3 ml of PBS plus 2% FCS plus 0.01% azide and surface-stained for 15 min with anti-human CD3-PerCP-Cy 5.5 and anti-human CD4-PE or CD8-R-PE (BD Biosciences). The cells were washed as above and developed with 100 μl/well 5-bromo-4-chloro-3-indolyl phosphate/NBT alkaline phosphatase (Moss) for 20 min at room temperature. The reaction was stopped by rinsing the plates with tap water. The numbers of spots were counted and expressed as the mean count ± SD. Anti-mouse IFN-γ Ab (mAb AN18; Mabtech) and biotinylated anti-mouse IFN-γ Ab (mAb R4–6A2–bio; Mabtech) were used in mice ELISPOT assay.
4°C. After washing, the cells were analyzed by FACScan (BD Biosciences). The data were reanalyzed using the WinMDI version 2.8 software package (J. Trotter, The Scripps Institute, San Diego, CA). The background in the negative cells was 0.1–0.4%. The background in cells stimulated with the rVV control was 0.6–1.5%. The percentages of specific CD8+/H11001+/IFN-/H9253+ were calculated by subtracting nonspecific CD8+/H11001+/IFN-/H9253+ cell frequencies from those stimulated with the rVV control. The percentages of specific CD4+/perforin were calculated by subtracting nonspecific CD4+/perforin cell frequencies from those stimulated with the rVV control.

**Ki67 expression and T cell phenotype**

PBMCs from vaccinated and unvaccinated animals were isolated and stimulated with a pool of HIV-1 peptides. Sixteen to 20 h after incubation, cells were washed with PBS, 2% FCS, 0.01% azide, and then surface-stained with anti-human CD3-allophycocyanin-Cy7 and CD4-PerCP mAbs. The cells were fixed, permeabilized (BD Biosciences), and stained with the human Ki67-PE-labeled mAb (isotype mouse IgG1, k) or with isotype control (BD Biosciences). The frequency of Ki67+ T cells was analyzed using a FACSCanto flow cytometer (BD Biosciences). PBMCs were also stimulated with or without the pool of HIV-1 peptides and the proportions of central and effector memory CD4 and CD8 T cells were assessed by the following Abs: CD95-FITC, CD28-PE, CD4-PerCP, CD8-PE-Cy7, CD3-allophycocyanin-Cy7, and IFN-α-allophycocyanin (cytomolgus, rhesus, and baboon reactivity, isotype mouse IgG1, k; BD Biosciences).

**Ab binding**

Total IgG and IgA titers were determined by ELISA as described previously (16). Briefly, 96-well EIA/RIA TM Stripwell plates (Corning) were coated overnight at 4°C with HIV-1 recombinant proteins or the pool of HIV-1 peptides at a concentration of 0.3 g/well. The plates were washed six times with 0.5% Tween 20/PBS, and blocked with 1% BSA (fraction

**FIGURE 2.** HIV-1-specific T cell immune responses detected by IFN-γ ELISPOT assay expressed as SFC per million splenocytes in immunized mice. Four mice per group were immunized four times s.c. with the candidate HIV-1 vaccines (lipidated, nonlipidated, or mixture of peptides) or adjuvant alone. Ten days after the last immunization, the mice were euthanized and ELISPOT assay was performed. The data are the mean ± SD values of duplicate samples from four mice in each group. *, Statistically significant differences with p ≤ 0.01–0.05.

**FIGURE 3.** HIV-1-specific CD8+ T cell response detected by ICS in the immunized macaques. PBMCs from vaccinated and control animals were stimulated with a pool of HIV-1 peptides, rVVs expressing HIV-1 proteins from subtypes A–F or empty rVV. After stimulation, cells were surface stained for CD3+ and CD8+, as well as stained intracellularly for IFN-γ and analyzed by flow cytometry. The results were analyzed with the WinMDI program. A, Dot plots show results from individual representative macaque. B, Percentage of CD8+IFN-γ to HIV-1 subtypes A–F is shown in each vaccinated macaque after subtraction of values from empty vaccinia. No specific CD8+ T cell response has been observed in control animals. ND, Not detected; Nd, not determined.

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V)PBS. Serial dilutions of cynomolgus plasma or saliva in 1% BSA (fraction V)/PBS were incubated overnight at 4°C. Plates were washed before the addition of a 1/2000 dilution of peroxidase-labeled affinity purified goat anti monkey IgG or IgA (Kirkegaard & Perry Laboratories). After washing, color was developed with O-phenylenediamine dihydrochloride (Sigma-Aldrich). The color reaction was stopped with 1 N HCl and absorbance was read at 490 nm with an ELISA plate reader (Bio-Rad).

Neutralization assays

Plasma from immunized macaques was collected in EDTA-coated tubes and neutralization assays were performed at Duke University (17) and Monogram Biosciences (18) as previously described. Briefly, HEK-293 cells were cotransfected with an HIV Env expression vector and a genomic HIV vector which contains a luciferase gene in place of Env. Viral stocks were collected from the HEK-293 cells and inoculated for 18 h with serial dilutions of heat-inactivated animal plasma (1/10 initial dilution, 3-fold serial dilutions). After incubation, the virus/plasma mixture was used to infect CCR5+/CD4+/CD95−/U87 cells. Virus infectivity was determined 3 days later by measuring the amount of luciferase activity expressed in infected cells. Neutralizing activity was reported as the concentration or dilution of each plasma required to inhibit the virus by 50% (IC50). The assay control for nonspecific inhibition was HIV pseudotyped with the non-HIV amphoMuLV Env. Plasma titers that were at least three times higher than those observed against the negative control virus (aMLV) were considered positive. Other control plasmas were NL43 and JRCSF, a laboratory-adapted X4-tropic strain and a neutralization-resistant R5-tropic primary isolate, respectively. HIV-1-positive serum (Z23) with a broad neutralizing capacity was used as a control.

Challenge study

Six- to 8-wk-old HLA-A2.1 mice (n = 4) were immunized with a mixture of lipidated and nonlipidated peptides. A total of 120 µg of vaccine were resuspended in sterile saline and mixed at a 1:1 ratio with Montanide ISA-51 adjuvant. The vaccine was injected s.c. with 1-mo interval between each immunization. Four weeks after the last immunization, mice were challenged i.p. with 2 × 106 PFU of a recombinant vaccinia virus expressing Gag/Pol/Env proteins from a subtype B variant of HIV-1. Mice were monitored for body weight, sickness, and abnormalities. On day 5, mice were euthanized with CO2 gas and the ovaries, the right lung, and spleens were dissected. Ovaries were homogenated separately using a glass Teflon homogenizer, centrifuged, and the supernatant was collected. Viral load was determined by performing plaque assays using the homogenated organs. Homogenate dilutions ranging from 103 to 106 were used to inoculate HeLa cells, which were incubated at 37°C for 3 h. Cells were washed once and covered with 2 ml of IMDM containing 10% FCS and 0.5% agarose. The plates were left at room temperature for 30 min and transferred to 37°C for 7 days. The plates were fixed overnight with 4% paraformaldehyde, stained with 0.05% neutral red in PBS, rinsed with tap water, and left inverted at room temperature to dry before counting.

Statistical analysis

For flow cytometry analysis, lymphocytes were gated on forward (FSC) vs side scatter (SSC) and 30,000–50,000 events were collected. CD8+, CD4+, and DP CD4+CD8+ T cells were identified based on SSC and positive surface expression of CD3. Cells were then gated based on CD8+, CD4+, CD8+CD4+ T cells vs perforin or IFN-γ. Furthermore, cells were gated based on CD28 and CD95 expression to define memory T cell subpopulations. Analysis was performed with the FACSCanto analyzer. Absolute numbers were calculated by multiplying the percentage determined on flow cytometry by the total lymphocyte count determined from a CBC analyzer. For the cell proliferation assay, results were expressed as stimulation index (SI) calculated by dividing the mean cpm of cells incubated with HIV-1 proteins by the cpm of cells incubated with medium alone. Results of ICS, Ab titer, and lymphocyte proliferation were expressed as mean ± SD. The t test was applied for the statistical analysis of the data.

Results

Vaccine design and synthesis

To elicit immunity capable of recognizing divergent stains of HIV, we used a novel approach to solid-phase peptide synthesis to obtain degenerative peptide mixtures representing major antigenic variants present in the five hypervariable regions of the HIV-1 gp120 as well as two variable epitopes present in Gag. Individual peptides ranged from 15 to 27 aa in length. The complete vaccine contained 7 lipidated and 5 nonlipidated peptide mixtures (96 lipidated variants and 80 nonlipidated variants) comprising a total of 176 unique peptide variants (Fig. 1A). At the completion of each

**FIGURE 4.** HIV-1 specific CTL responses detected by IFN-γ ELISPOT assay expressed as SFC per 1,000,000 PBMCs in immunized macaques. PBMCs from vaccinated and control animals were stimulated with rVVx expressing HIV-1 genes from subtypes A–F or empty rVV. The number of SFC induced was calculated by subtraction of SFC of cells stimulated with control rVV from SFC of cells stimulated with rVV-HIV-1. The data are shown as the mean ± SD. *, Statistically significant difference with p ≤ 0.01–0.05.
synthesis reaction, either 8 or 16 peptide variants were expected to be present due to incorporation of 2 aa in the growing peptide chain at three or four defined positions. To ensure consistency, HPLC was performed on each batch of peptides. A representative HPLC histogram of two different batches is shown in Fig. 1B. Using mass spectrometry, we were able to identify the appropriate number of unique peptide species with their anticipated m.w. (Fig. 1C).

A portion of each of the seven peptide mixtures was lipidated with palmitic acid. Two of the peptide mixtures, which represent variable epitopes located in Gag, were lipidated entirely (Fig. 1A). The lipidated form of the peptide variants served two functions: to elicit CTL responses and to activate innate immunity during vaccination (19–21). Indeed, we vaccinated mice with nonlipidated, lipidated, or a combination of both lipidated and nonlipidated peptide mixtures, and evaluated cellular immunity using an IFN-γ/H9253 ELISPOT assay (Fig. 2). We observed that immunization with a combination of both lipidated and nonlipidated peptides induced more robust cellular immunity than did lipidated or nonlipidated peptides alone.

**Vaccination strategy**

Based on the results in mice, we chose a vaccine formulation containing both lipidated and nonlipidated peptides to test the immune response induced in nonhuman primates. Six cynomolgus macaques were immunized with seven lipidated and five nonlipidated peptide mixtures at a 1:1 ratio with Montanide ISA-51 adjuvant. The vaccine was administered five times at months 0, 1, 3, 7, and 10. Macaques were bled 10–14 days after each vaccination and the immunogenicity of the vaccine was compared with control macaques.

**A multivalent HIV-1 peptide vaccine elicits a broad cross-subtype T cell immune response in nonhuman primates**

Cross-subtype reactivity of specific CD8+ T cell immune response was determined with ICS and ELISPOT assays. PBMCs from immunized macaques were cultured and stimulated with a panel of recombinant vaccinia viruses representing the most predominant HIV-1 subtypes A–F. After stimulation, cells were surface stained for CD3 and CD8, as well as stained intracellularly for IFN-γ and analyzed by flow cytometry. Following each immunization with

| Table I. PBMCs from immunized macaques were cultured and stimulated with the HIV-1 pool peptides after the first, third, fourth, and fifth vaccination |  |
|---|---|---|---|---|---|---|---|---|
| Number of Vaccination | SI in Immunized Macaques | No. of Positive SI/No. of Tested Macaques |
| 1 | 013 | 011 | 079 | 059 | 020 | 012 |
| 2 | Nd | Nd | Nd | Nd | Nd | Nd | 0/6 |
| 3 | 6 | 9 | 6 | 2 | 7 | 4.5 | 5/6 |
| 4 | 6 | 5.8 | 8 | 6 | 5 | 8.5 | 6/6 |
| 5 | 2 | 4.5 | 4.6 | 4 | 8.5 | 12 | 6/6 |

*a Five days later, [3H]thymidine was added and after 16 h the incorporated radioactivity was measured in harvested cells. SI was calculated by dividing the mean cpm of cells incubated with HIV-1 peptides by the cpm of cells incubated with medium alone. SI was equal to the cutoff value (dash line) after the first vaccination. Nd, Not determined.

**FIGURE 5.** T cell proliferation results in macaques immunized with the candidate vaccines. PBMCs were cultured and stimulated with the recombinant proteins HIV-1 MN gp120 (subtype B), HIV-1 IIIB gp160 (subtype B), HIV-1 CM gp120 (subtype E), HIV-1 96ZM651 gp120 (subtypes C), HIV-1 SF162 gp120 (subtype B), and HIV-1 93TH973 gp120 (subtypes A/E). Five days later, [3H]thymidine was added and after 16 h the incorporated radioactivity was measured in harvested cells. Results were expressed as SI calculated by dividing the mean cpm of cells incubated with HIV-1 proteins by the cpm of cells incubated with medium alone. Each bar represents SI from an individual immunized macaque. The dotted line indicates the cutoff value which is a SI of 2.

**FIGURE 6.** Measurement of cytotoxic CD4+ T cell activity in immunized macaques. Two weeks after the last immunization, PBMCs from vaccinated and control animals were stimulated with rVVs expressing HIV-1 proteins from subtypes A–F or empty rVV. After stimulation, cells were surface stained for CD3+ and CD4+, as well as stained intracellularly for perforin and analyzed by flow cytometry. Frequency of perforin-positive CD4+ T cell was detected after stimulation with HIV-1 subtypes B, C, and E and not other HIV-1 subtypes. The data are shown as the mean ± SD. *, A significant difference between the immunized group and control (p ≤ 0.05).
the candidate HIV-1 vaccine, cellular immunity was analyzed. Notably, five of six immunized macaques showed a high frequency of cross-subtype IFN-γ-secreting CD8+ T cells to all HIV-1 subtypes A–F after the last immunization (Fig. 3). To confirm the results obtained by ICS, ELISPOT analysis was conducted using PBMCs from immunized macaques. PBMCs from vaccinated and unvaccinated animals were stimulated with rVVs expressing HIV-1 genes from subtypes A–F or empty rVV. The number of spot-forming cells (SFC) induced was calculated by subtraction of SFC from cells stimulated with rVV-HIV-1 from SFC from cells stimulated with control rVV. PBMCs from immunized animals produced a statistically significant increase in the number of spots detected in response to stimulation with heterologous subtypes A–F compared with unstimulated cells (Fig. 4). These results demonstrate that our multivalent vaccine is capable of eliciting a broad cross-specific CD8+ T cell response to HIV-1 variants despite MHC diversity between animals and significant amino acid sequence differences in tested HIV-1 isolates.

Induction of proliferative immune response and cytotoxic CD4+ T cell activity by multivalent HIV-1 peptide regimen in macaques

Proliferation of CD4+ T cells plays a crucial role in regulation of both humoral and cellular immune responses by expansion of Ag-stimulated B and CD8 T cells, respectively. PBMCs from immunized macaques were stimulated with HIV-1 Env proteins derived from three subtype B variants, one subtype E variant, one subtype C variant, and one subtype A/E variant. Five days after stimulation, [3H]thymidine was added and 16 h later, cells were harvested and thymidine incorporation was measured. Two weeks after the fifth immunization, two macaques showed response to variants from all three subtypes. One animal responded to variants from subtypes B and E, and two additional animals responded to multiple subtype B variants (Fig. 5). One of the immunized macaques responded weakly to the Env proteins tested at this time point, but did respond to subtypes B and E when assessed at an earlier time point (4 wk after the third immunization, data not shown). In addition, proliferative immune responses to HIV-1 pool peptides were also detected in five of six vaccinated macaques after the third immunization. All six macaques developed proliferative responses to the pooled HIV-1 peptides; however, the level of T cell proliferation was decreased in four and increased in two animals (Table I).

It has previously been shown that CD4+ T cells with cytotoxic activity are expanded during chronic viral infections (22), and can be induced with vaccination (23). Analysis of PBMCs 2 wk after the last immunization demonstrated a high frequency of perforin-positive cytotoxic CD4+ T cells when stimulated with HIV-1 subtypes B, C, and E (Fig. 6). None of the immunized animals showed a perforin-positive CD4+ T cell response to HIV-1 subtypes A, D, or F. Perforin-positive cells were not detected at the later 6 wk time point, consistent with the notion that these cells are a terminally
differentiated, effector population of Th cells that are lost over time (24).

**Immunophenotype characterization of specific memory cells after vaccination with HIV-1 peptide regimen**

The induction of long-lived specific cell-mediated immune responses is a critical aspect in the development of an effective vaccine. Memory T cells play an important role in jeopardizing viral replication and in providing long-term immunity (25–27). Therefore, it was of interest to measure the induction of these cells in response to immunization. We characterized specific HIV-1 memory T cell responses after the final immunization. The surface markers CD28 and CD95 have been used to distinguish different populations of memory T cells (28, 29). Immunophenotype characteristics of specific memory cells were evaluated upon stimulation with specific HIV-1-pooled peptides. As indicated in Fig. 7B, the candidate vaccine was able to generate a significantly higher level of effector (EM) and central (CM) memory DP CD4+CD8+ T cell in immunized macaques compared with control animals. In addition, specific EM cells were increased in both single-positive (SP) CD4+ and CD8+ T cells after the last immunization. No significant difference between SP CM CD4+ and CD8+ T cells was detected between immunized and control macaque groups.

Previous studies have shown that the Ki67 molecule plays important roles in cellular survival and proliferation. Notably, it is not expressed in resting cells. Thus, this marker can be used to monitor cell cycle progression. We evaluated the expression of Ki67 pre-immunization and after the first, third, and fifth immunization in macaques. Four animals (079, 012, 0120, and 059) had higher T cell frequencies of Ki67 expression after the last vaccination. There was no expression of Ki67 in animal 011 before or after vaccination (data not shown). The assay was not performed in animal 013 due to lack of available PBMCs.

**Induction of cross-subtype humoral immunity in immunized macaques**

To measure Ab titers against various HIV-1 gp120 proteins, both pre- and postimmunization plasma samples from the immunized macaques were collected, and binding reactivity to recombinant HIV-1 Env proteins MN, SF162, IIIB, CM, and the pool of HIV-1 peptides were determined by ELISA. A significant enhancement of the levels of anti-Env or anti-HIV-1 peptide Abs was observed in all immunized animals (Fig. 8A). In fact, the magnitude of Ab response augmentation in macaques was even higher than in mice that were immunized with the same HIV-1 vaccine regimen (data not shown). The kinetics of Ab responses in sera of immunized animals were also characterized using HIV-1 Env from isolates MN, 96ZM651, CM, and 93TH975 (Fig. 8B).

Furthermore, we determined whether the multivalent HIV-1 vaccine was able to augment mucosal immunity. Saliva IgA was tested using HIV-1 subtype B (IIIB and MN) and one subtype E (CM) Env proteins. Three of six macaques (079, 011, and 020) had IgA titers against at least one Env protein; two macaques (013 and 012) had titers against both HIV-1 subtypes B and E Env proteins. HIV-1 Env IgA Ab titers were between 1:50 to 1:300.

To determine whether vaccine-induced Abs had functional, biologically relevant neutralizing activity, sera from the vaccinated animals were assessed for their ability to neutralize a panel of both TCLA and primary X4 and R5 viruses. Neutralizing activity of sera was analyzed at Duke University and Biosciences Monogram (former ViroLogic), against a total of 3 TCLA and 14 primary isolates of HIV-1 variants, respectively. The principle in all Nab assays used both, in Duke or Monogram, was based on reductions in viral infectivity in target cells expressing both CCR5 and CxCR4 coreceptors. Neutralizing Ab activity was based on the serum dilution at which relative luminescence units (RLU) were reduced by 50% compared with virus control wells. Although our vaccine was designed mainly based on CD4+ and CD8+ T cell epitopes, neutralizing activity was detected in all immunized macaques against HIV-1 isolates MN, SF162, and SS1196.01 after the fifth immunization (Fig. 9). The kinetics of Nab response was further tested against 14 HIV-1 primary isolates. Three of six animals showed Nab response to HIV-1 subtype D isolates 92UG024 and 93UG059 after the third and fifth vaccination. Neutralizing response in macaque 079 against isolate 93UG059 reached a titer of 1:51 after the third immunization and increased to 1:62 after the
fifth immunization. Similarly, macaques 013 and 059 had persisting Abs after the third and fifth immunizations against HIV-1 subtype D isolate 92UG024 (Table II). One of six animals also showed Nab response to HIV-1 subtype E isolate 93TH053 after the third vaccination but this response was not detected later. Primary isolates from subtypes A–C were not neutralized by any of the macaque sera.

**FIGURE 10.** Challenge study in mice with rVV-expressing HIV-1 IIIB (subtype B). HLA A2.1 mice were immunized with the HIV-1 candidate vaccine, or adjuvant alone and challenged i.p. with 2 × 10^7 PFU of recombinant vaccinia virus expressing HIV-1 IIIB Env/Gag/Pol proteins after the last immunization. Five days later, mice were sacrificed and the ovaries were harvested and assayed for vaccinia virus titer by plaque assay on HeLa cell lines, stained with crystal violet, and plaques counted at each dilution. Viral loads were significantly lower in vaccinated mice compared with control group (p < 0.05). The data are shown as the mean ± SD.

**Table II.** Nab titers against HIV-1 subtypes D (92UG024 and 93UG059) and E (93TH053) in immunized macaques

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a Plasma from immunized macaques was obtained 2 wk after the first, third, and fifth vaccination and Nab activity was measured against HIV-1 primary isolates. The Nab titer is reported as the reciprocal of the dilution of plasma that produces 50% inhibition of target cell infection. A positive Nab assay (bold and underlined) was defined as an Ab titer at least three times higher than the negative control infection with aMLV. The titer of plasma against control strains (aMLV, NL43, and JRCSF) is shown. An HIV-1-positive serum (Z23) with a broad neutralizing capacity was used as a control serum. None of the immunized macaques were able to induce neutralizing activity against HIV-1 subtypes A, B, and C.

**Challenge of HLA A2.1 mice**

Because the vaccine was designed to elicit CTL responses to Env and Gag proteins of HIV-1, we were unable to evaluate its potential efficacy in the vaccinated monkeys; simian HIV (SHIV) viruses that could be used in challenge experiments express SIV-derived Gag, thereby precluding determination of the potential efficacy of vaccine-induced cellular immunity against the epitopes in this protein. Moreover, the use of SHIV challenge in the species of macaques is of questionable value. For instance, SHIV-89.6P and SIVmac251 exhibits only a low viral replication in cynomolgus compared with rhesus macaques (30). Due to the above limitations to measure vaccine-induced efficacy, a murine model was used. HLA A2.1 mice were immunized four times with the same vaccine formulation used in the macaques. Four weeks after the last immunization, mice was challenged i.p. with 2 × 10^7 PFU of recombinant vaccinia virus expressing HIV-1 IIIB (subtype B) Env/Gag/Pol proteins. Five days later, mice were sacrificed and the ovaries were harvested, as this is the organ in which vaccinia preferentially replicates. Vaccinia virus titers were evaluated by plaque assay using dilutions of ovary homogenates. As shown in Fig. 10, the group immunized with the HIV-1 multivalent peptides showed more than a 2-log reduction in titer of virus compared with control (p < 0.05). Although it is unclear how well efficacy in this challenge model may correlate with nonhuman primates or humans, the data nevertheless indicate that vaccine-induced immunity was able to recognize and eliminate processed viral Ags in vivo.

**Discussion**

The unprecedented HIV-1 mutation rate and resulting antigenic heterogeneity among viruses circulating throughout the world poses a significant challenge to vaccine development (31). Several strategies are currently being explored to circumvent the extensive antigenic variability of HIV-1. An immense deal of interest has
been focused on the induction of specific immunity based on conserved regions of HIV-1; however, none of these approaches were able to induce a broadly cross-reactive immunity and only a limited breadth of reactivity was raised against HIV-1 variants (32–35).

Previous studies have demonstrated that CD8+ T cell function is inversely correlated with viral load and is associated with protection and disease (36, 37). In an interesting study, Rowland-Jones et al. (38) showed the presence of a high frequency of CD8+ T cells in HIV-resistant sex workers in Nairobi. These results concluded that effective cross-subtype CD8+ T cells could be responsible for protection against constant HIV exposure and infection.

Advances in MHC-peptide binding studies and characterization of resulting antiviral immune responses has initiated a new era in vaccine design. Using lipidated peptide immunogens is one of several strategies in the improvement of immunogenicity that triggers TLR pathways. Jackson et al. (39) demonstrated that lipid moieties present on peptides prolongs the duration of Ag presentation, enhances cytosolic uptake of peptide immunogens, activates innate immunity due to TLR2 agonist effect, and differentiates nonactivated B cells into Ig-secreting plasma cells. In another study, Espuelas et al. (40) studied the effect of lipopeptide analogues incorporated into liposomes on the maturation of human DCs. They found that slight modifications in the peptide moiety of lipopeptides have an immense impact on cell surface markers such as CD80, CD83, CD86, and HLA-DR on human DCs. In a landmark study by Gahery et al. (41), a candidate HIV-1 vaccine comprising six lipidated peptides (Nef, Gag, and Env) were administered to highly active antiretroviral therapy-treated patients. The breadth and magnitude of HIV-1-specific cell-mediated immune responses were measured and notably, some patients were able to induce new CD4 and CD8+ T cell responses after vaccination.

It is believed that all CD8+ T cells are not identical in terms of their ability to eliminate virus-infected cells. Essential factors, such as avidity between TCR-MHC/peptide, frequency of effective CTL activity, or structural constraints on the epitope region, play imperative roles in CD8+ T cell effectiveness (42, 43). There are unanswered questions about the immune response generated from vaccines targeting conserved epitopes, which may not be immunodominant and whether the frequency of specific immune responses is not sufficient to control viral infection. In a peptide vaccine study, eight rhesus macaques were immunized with seven lipidated peptides comprising SIV-Nef and Gag proteins. Specific CD8+ T cells were detected in seven animals and two of eight animals demonstrated a multispecific CTL immune response (44). These animals plus three control macaques were further challenged with SIVmac251. The three control animals died on month 4 after infection. All vaccinated macaques became infected, but survived after week 35 postchallenge, having a 2- to 3-log decrease in viral load compared with the control animals (45).

Unlike conserved regions, hypervariable regions of HIV-1 contain highly immunogenic regions encompassing critical B and T cell epitopes. Although the highly variable HIV regions allow the virus to escape from the immune response, targeting the immunodominant epitopes in hypervariable regions may prove to be more effective in the induction of immunity with greater depth compared with the vaccines targeting conserved areas (46–48).

In this study, we demonstrate the ability of a polyvalent peptide-based vaccine targeting the variable regions of Gag and Env proteins to elicit broadly reactive T cell responses in immunized macaques. Although some amino acid positions within an epitope are quite variable, limits exist in the variation. For example, 5 or fewer amino acids comprise the majority of amino acids present at any given variable position, and there is little evidence for the presence of all 20 amino acids at any single position within an epitope. This somewhat limited diversity exists despite immune pressure targeting these regions. In this study, several peptide variants were designed to overcome the genetic diversity by providing frequently occurring amino acid substitutions of each epitope. The synthetic peptide variants first were tested in HLA-A2.1 mice alone or in combination. The outcome of the preliminary experiments in mice led us to immunize macaques with a mixture of 176 peptides, representing major variants known or predicted to be present in the hypervariable regions of HIV-1. To quantify cross-subtype CD8+ T cell recognition, PBMCs from immunized and control macaques were analyzed for IFN-γ production in response to HIV-1 subtypes from global geographical regions. The multivalent vaccine was able to elicit a broad cross-subtype CD8+ T cell response as measured by ICS. To verify these results, the cross-subtype reactivity of CD8+ T cells was further characterized by the ELISPOT assay. These results also revealed a striking cross CD8+ T cell response toward heterologous HIV-1 subtypes in all vaccinated animals. These results are significant as HIV-1 amino acid sequences within a subtype may differ by up to 20% and between subtypes by up to 35% within the regions that our vaccine targets.

To pursue the question of whether the vaccine regimen elicited CD4+ Th immune responses in macaques, a proliferation assay was performed using Env proteins from different HIV-1 variants as stimulating Ags. Proliferative immune responses were detected in five to six immunized macaques against HIV-1 Env from two or more distinct variants. The responses to Env proteins were different in each macaque, for instance, two of the macaques responded better to subtypes C and E than other subtypes. The heterogeneity of these responses may be because macaques are outbred and have diverse MHC polymorphisms and carry different TCR repertoires. This may also explain variability in the response of vaccinated animals to the panel of recombinant Env proteins. MHC diversity and TCR variability are also challenges to HIV vaccine development in the human population (49).

Consistent with induction of a reactive Th cell repertoire, CD4+ T cells were further analyzed for secretion of perforin. Previous studies showed that perforin-positive cytotoxic CD4+ T cells could contribute to the breadth of immune response by forming pores in membrane of target cells that facilitate the entry of granzymes and consequently initiate an apoptotic cascade pathway (50, 51). Analysis of perforin-positive cytotoxic CD4+ T cells 2 wk after the fifth immunization demonstrated an increased frequency to HIV-1 subtypes B, C, and E but not A, D, or F. Interestingly, perforin-positive CD4+ T cells were not detected at the later 6 wk time point, indicating that cytotoxic CD4+ T cells are time dependent and that their population decreases over time (52).

In the quest for an effective vaccine, memory T cells may be imperative to viral destruction and providing long-term immunity (53–56). CM and EM T cells are recognized as two main populations of memory T cells with different migration patterns (57). In particular, CM T cells expressing CD28+, CD95+, CCR7+, and L-selectin, which are homing on lymph nodes, whereas EM T cells do not express CD28 or CCR7 and home to peripheral tissues (58, 59). The mechanism behind the generation of memory T cells is not understood; however, the type and potency of Ags may influence the quantity and differentiation of memory T cells produced in response to Ags (59). In this study, an extensive analysis of specific memory T cell lymphocytes was performed in control vs vaccinated macaques after the last immunization. Our results indicated that effector memory SP CD4+ and CD8+ T cells (p < 0.05 and 0.008 respectively) are up-regulated after vaccination. In addition, we found that central (CD28+CD95+) and effector
Expression of Ki67 is important in differentiation between resting and activated T cells. Furthermore, induced Ki67 may correlate with cell cycle progression and IL-2 secretion. Thus, we further analyzed the kinetic expression of Ki67 before and after the first, third, and fifth vaccination. Expression of Ki67 by T cells was detected in four of five animals. The results demonstrate that our candidate vaccine may be able to generate specific memory T cell immune responses that may contribute to long-term protection.

To address whether our vaccine induced a similar depth and breadth in the humoral response, the binding Ab titer and neutralizing activity was characterized in immunized macaques. Two weeks after each vaccination, plasma was collected and Ab titer was measured against a panel of Env proteins and a mixture of HIV-1 peptides. All animals showed a substantial level of IgG Ab titer to the selected immunogens after the second immunization as measured by ELISA. Given that HIV infection predominately occurs via the mucosal route, there is interest in developing candidate HIV vaccines that can elicit mucosal immunity (60–62). To study this phenomenon, saliva from immunized macaques was collected and anti-Env IgA Ab titer was measured against proteins from distinct HIV-1 subtypes. Interestingly, mucosal immunity was detected in immunized animals even though the candidate vaccine was administered s.c.

Induction of Nabs is also important in an effective vaccine. We first determined whether Nab directed against TCLA strains of HIV was detectable. Positive neutralizing activity was observed against HIV-1 MN, SF162, and SS1196 isolates. We also measured Nabs raised against 14 HIV-1 primary isolates from subtypes A–E. Notably, three of six immunized macaques developed neutralization activity against HIV-1 subtype D isolates 92UG024 and 93UG059. One macaque was also able to induce neutralizing activity against HIV-1 subtype E (93TH053) after the third vaccination. HIV-1 primary isolates from subtypes A–C were not neutralized by any of the macaque plasma. The Env peptides incorporated in our vaccine formulation included the variable regions (V1 to V5). Except for an area of 4 aa (IGPG) in the tip of the V3 loop, which is conserved between HIV-1 subtypes (63), most other peptide regions demonstrated variability. To compare the similarity between our peptide sequences with known B cell epitopes in the HIV-1 isolates demonstrating neutralization, HIV-1 B cell epitopes sequences were searched in the Los Alamos database. We found that 5 aa (FYKDL) in the V2 peptide and 5 aa (RKSIR) in the V3 peptide sequence are known B cell epitopes; however, it is not clear whether these sequences have been associated with neutralizing anti-HIV-1 activity.

To our knowledge, this is the first time that Nabs are demonstrated against HIV-1 isolates using linear hypervariable epitopes as immunogens. Because little is known about the kinetics of the antiviral immune response with this immunization approach, macaques were immunized five times. In contrast to the T cell response plateau observed after the third vaccination, Nab titers increased after the fourth and fifth booster immunizations.

We were not able to evaluate the potential efficacy of vaccine by SIV challenge due to highly divergent sequences between the Env genes of HIV-1 and SIV. Recent studies showed that chimeric SHIV strains are not an appropriate model to test the efficacy of HIV-1 vaccines and that the results have the potential to be misinterpreted (64–67). To acquire virulence in nonhuman primates, SHIV strains have been passed several times through animals. These passages result in numerous alterations in the SHIV Env protein compared with the HIV-1 Env protein, and accordingly limit their use as a challenge model for HIV vaccines.

Additionally, the natural mechanism of these chimeric viruses limits their relevance in appraisal of CTL-based vaccines. Pathogenic SHIV89.6P has been shown to induce a severe loss of CD4+ T cells, while such a rapid loss of circulating CD4+ T cells is unusual in HIV-1 individuals (68). Unlike SHIV89.6P, some pathogenic SIV strains such as SIVmac251 or SIVsmE660 do not downregulate circulating CD4+ T cells and replicate highly in the chronic phase of disease, again being different from the HIV-1 mechanism of pathogenesis (69). Mechanisms of chimeric viruses are dependent on the species of macaques. For instance, viral replication of SHIV-89.6P and SIVmac251 in cynomolgus is lower than either Chinese or Indian rhesus macaques and CD4+ T cell loss is lower and survival is longer in cynomolgus and Chinese rhesus macaques than Indian rhesus macaques (70). Due to these difficulties in macaques challenge, a mouse model was chosen to evaluate efficacy of the vaccine. HLA-A2.1 mice were immunized with the vaccine, followed by challenge with a recombinant vaccinia virus expressing HIV-1 IIIB Env/Gag/Pol proteins. The results indicate that vaccine-induced immunity was able to recognize and eliminate processed viral Ags in vivo. Taken together, these results indicate that our multivalent peptide vaccine is able to induce moderate humoral but broadly reactive cellular immune responses with both breadth and depth against HIV-1 variants from different global geographical regions. This approach is now set to undergo the first human clinical trial on HIV-1-infected individuals in Ottawa to examine its safety and immunogenicity.

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