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Development of a DNA Vaccine Designed to Induce Cytotoxic T Lymphocyte Responses to Multiple Conserved Epitopes in HIV-1

Cara C. Wilson,* Denise McKinney,† Michelle Anders,* Samantha MaWhinney,* Jeri Forster,* Claire Crimi,† Scott Southwood,† Alessandro Sette,2† Robert Chesnut,† Mark J. Newman,† and Brian D. Livingston3†

Epitope-based vaccines designed to induce CTL responses specific for HIV-1 are being developed as a means for addressing vaccine potency and viral heterogeneity. We identified a set of 21 HLA-A2, HLA-A3, and HLA-B7 restricted supertype epitopes from conserved regions of HIV-1 to develop such a vaccine. Based on peptide-binding studies and phenotypic frequencies of HLA-A2, HLA-A3, and HLA-B7 allelic variants, these epitopes are predicted to be immunogenic in greater than 85% of individuals. Immunological recognition of all but one of the vaccine candidate epitopes was demonstrated by IFN-γ ELISPOT assays in PBMC from HIV-1-infected subjects. The HLA supertypes of the subjects was a very strong predictor of epitope-specific responses, but some subjects responded to epitopes outside of the predicted HLA type. A DNA plasmid vaccine, EP HIV-1090, was designed to express the 21 CTL epitopes as a single Ag and tested for immunogenicity using HLA transgenic mice. Immunization of HLA transgenic mice with this vaccine was sufficient to induce CTL responses to multiple HIV-1 epitopes, comparable in magnitude to those induced by immunization with peptides. The CTL induced by the vaccine recognized target cells pulsed with peptide or cells transfected with HIV-1 env or gag genes. There was no indication of immunodominance, as the vaccine induced CTL responses specific for multiple epitopes in individual mice. These data indicate that the EP HIV-1090 DNA vaccine may be suitable for inducing relevant HIV-1-specific CTL responses in humans. The Journal of Immunology, 2003, 171: 5611–5623.

Infection with HIV-1 results in a disease state characterized by progressive immune dysfunction, ultimately resulting in AIDS in the majority of infected individuals. Although disease progression occurs in the presence of HIV-1-specific immune responses, there is evidence that cellular immune responses, specifically those mediated by CD8+ CTL, can contribute to the control of HIV-1 replication. The antiviral effects of virus-specific CTL were initially demonstrated through the observed temporal association between CTL responses and HIV-1 disease progression. In acute HIV-1 infection, an early expansion of CD8+ CTL specific for HIV-1 structural and regulatory gene products was observed several weeks after infection. This expansion of CTL was associated with the initial decline of HIV-1 plasma viremia (1, 2). Similarly, clinical long-term nonprogressors are characterized by the presence of low viral loads, slower declines in CD4+ T lymphocyte counts, and broadly reactive CTL responses, supporting the belief that CTL responses may help to control viral replication in these chronically infected individuals (3–11).

Direct experimental evidence demonstrating the importance of CTL for controlling lentiviral infections was developed using the SIV infection and pathogenesis model with rhesus macaques or pathogenetic strains of the hybrid simian HIV-1. In these studies, in vivo elimination of CD8+ T lymphocytes in animals during chronic SIV infection resulted in a marked increase in viremia and associated pathogenesis (12, 13). Induction of virus-specific CTL responses using vaccines has also been reported to contribute to control of SIV replication, and associated pathogenesis, in multiple independent studies (14–17). The effectiveness of CTL control was clearly documented by the finding that SIV mutants with alterations to dominant CTL epitopes could escape CTL recognition, resulting in increased viral replication and accelerated disease progression (18–21). It is interesting to note that although escape from immunological control was often mediated through a change in a single dominant CTL epitope, suggesting that CTL responses to a single epitope were mediating the protective effect, the induction of a CTL response against a single epitope using vaccination strategies has proved insufficient for controlling viral replication in vivo (22). Taken together, data obtained from the simian HIV-1/SIV rhesus macaque model and human natural infection studies strongly suggest an important role for HIV-1-specific CTL in controlling HIV-1 replication and delaying disease progression.

Vaccines based on CTL epitopes represent a logical approach to generate effective cellular immunity in both the prophylactic and therapeutic settings because multiple epitopes can be incorporated into the vaccine design with the goal of inducing broadly reactive responses composed of multiple CTL clones directed against different epitopes. Epitope selection can be biased toward those that are most highly conserved among viral types and subtypes, and both dominant and subdominant epitopes from numerous viral gene products can be used. Although epitope-based vaccines are

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often thought to be limited with respect to HLA polymorphism and population coverage, the use of supertype-restricted epitopes, those capable of binding with significant affinity to multiple related HLA alleles, provides a means to address this problem. The use of epitopes capable of binding alleles representing as few as three HLA superfamilies, HLA-A2, HLA-A3, and HLA-B7, results in predicted recognition in nearly 90% of the global population, regardless of ethnicity (23).

Highly defined CTL epitopes can be incorporated into vaccines using different formats and delivery methodologies. Epitopes derived from HIV-1 and used in the form of synthetic peptides formulated with clinically acceptable IFA (ISA-51) were evaluated in phase 1 clinical trials, and measurable CTL responses were induced in a subset of volunteers (24, 25). A vaccine consisting of multiple synthetic peptides conjugated to lipids was also shown to be immunogenic in a clinical trial (26). Unfortunately, lipid- and emulsion-based vaccine formulations can be difficult to manufacture and are often toxic, causing site-of-injection irritation. The number of epitopes that can be incorporated into a single formulation is also limited, which may negatively impact population coverage and the ability to induce broadly reactive CTL responses.

Vaccine delivery approaches suitable for use with large numbers of CTL epitopes include DNA plasmid and viral vector formats. The delivery of highly defined CTL epitopes derived from lymphocytic choriomeningitis virus using a DNA vaccine format resulted in the induction of protective cellular immune responses (27, 28). Studies using other pathogen model systems have shown that experimental DNA plasmid vaccines encoding minimal CTL epitopes can be used to simultaneously induce CTL responses against multiple epitopes (29–33). DNA plasmid vaccine and a viral vector construct based on modified vaccinia Ankara virus have been used either singly or in combination to induce CTL responses against SIV, HIV-1, and Plasmodium falciparum epitopes (34–39), and clinical trials are planned or ongoing (35, 36). Thus, the DNA plasmid and viral vector vaccine formats appear to be well suited for use with multiple CTL epitopes.

We developed a DNA plasmid vaccine, designated EP HIV-1090, encoding 21 well-defined and highly conserved CTL epitopes derived from both structural and regulatory/accessory proteins of HIV-1. In this work, we describe the process used to identify and characterize these 21 CTL epitopes with respect to their HLA-binding properties, their sequence conservation in diverse viral types and subtypes, their estimated population coverage, and their antigenicity in HIV-1-infected humans. The EP HIV-1090 DNA vaccine was tested in HLA transgenic mice to document the immunogenicity of multiple epitopes and the ability of vaccine-induced CTL to recognize human target cells expressing intact HIV-1 Gag and Env. These data represent a portion of the preclinical data developed to support phase 1 clinical testing of this experimental vaccine in HIV-1-infected and uninfected volunteers.

Materials and Methods
Identification of HIV-1 vaccine candidate epitopes based on HLA-peptide-binding motifs

Intact HIV-1 sequences in the Los Alamos database were analyzed using text string search software to identify amino acid sequences of 8–11 aa in length containing the HLA-A2, HLA-A3, or HLA-B7 supertype motifs (Table I) (40). The analysis included complete sequences from 64 HIV-1 isolates from the following subtypes: 3 A, 18 B, 8 C, 4 D, 2 F, 3 G, 3 H, 2 J, 1 N, 2 0, and 18 circulating recombinant forms. Nine HIV-1 gene products, Gag, Pol, Env, Nef, Rev, Tat, Vif, Vpr, and Vpu, were scanned for motif-bearing amino acid sequences. Those sequences containing HLA-A2 or HLA-A3 motifs, in which the contiguous amino acid sequences were identical in ≥50% of the 18 subtype B isolates in the data set and commonly found in isolates from other subtypes, were selected for further analyses. Similarly, 9–10 aa sequences that were positive for the HLA-B7 motif and present in ≥30% of the subtype B isolates were identified.

Measurement of epitope peptide binding to solubilized HLA class I molecules

Synthetic peptides representing potential CTL epitopes were synthesized using an Applied Biosystems (Foster City, CA) 430A peptide synthesizer and FmOC chemistry (41, 42). After synthesis, peptides were cleaved from the resin, the protecting groups were removed, and peptides were purified by reverse-phase HPLC. The purity of the peptides was typically greater than 95%, determined by mass spectrometry and/or composition analysis.

Competitive binding measurements, based on the use of radiolabeled reference peptides known to bind to the selected HLA molecules with high affinity, were used to determine peptide-binding affinity to solubilized HLA-A2, HLA-A3, and HLA-B7 supertype molecules (43, 44). Peptides were initially screened for binding to the prototype HLA superfamily allele (Table I). Peptides that bound with an affinity of less than 500 nM were subsequently tested for the capacity to bind additional supertype alleles. An affinity threshold of 500 nM generally correlates with the capacity of a peptide to elicit a CTL response; accordingly, this value was utilized as a criterion for epitope prediction.

To measure the binding of HIV-1 test peptides to HLA molecules, 5–50 nM of the purified HLA was incubated with 0.012–120 μg/ml of test peptide in the presence of 1–10 nM 125I-radiolabeled standard peptides for 48 h in PBS containing 0.05% Nonidet P-40 in the presence of protease inhibitors at pH 7.0. Following incubation, HLA-peptide complexes were separated from free peptide by gel filtration on 7.8 mm × 15-cm TSK200 columns (TosoHaas 16215, Philadelphia, PA) with PBS, pH 6.5, containing 0.5% Nonidet P-40 and 0.1% NaN3. The eluate from the TSK columns was passed through a Beckman 170 radioisotope detector (Fullerton, CA), and the fraction of the reference peptide bound to HLA protein was determined. In appropriate stoichiometric conditions, the concentration of test peptide needed to displace 50% of the bound reference peptide (IC50) represents a reasonable approximation of the true affinity of interaction (Kd).

Calculation of potential population coverage

Potential population coverage for the EP HIV-1090 DNA vaccine was calculated using reported gene frequencies for HLA-A and HLA-B alleles (45–47). For analyses in which frequency data were not available at the

<table>
<thead>
<tr>
<th>Supertype</th>
<th>Motif*</th>
<th>Prototype Allele</th>
<th>Verified Supertype Allelesa</th>
<th>Predicted Supertype Allelesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7</td>
<td>X (P) Xα–γ (ALIMVFWY)</td>
<td>B*0702</td>
<td>B<em>3501–08, B</em>5101–5, B<em>5301, B</em>5401, B<em>0703–05, B</em>1508, B<em>5501–02, B</em>6501–02, B<em>6701, B</em>7601</td>
<td>B<em>1511, B</em>4201, B*5901</td>
</tr>
</tbody>
</table>

* Individual amino acids defining the motifs used for epitope identification are shown as an X, Xα–γ, is shown to indicate that the motif anchor positions, shown in parentheses are separated by 6–7 aa. Any single amino acid contained within parentheses is acceptable at the motif anchor position.

* Verified alleles include alleles whose specificity has been determined by pool sequencing analysis, peptide-binding assays, or analysis of the sequences of CTL epitopes.

* Predicted alleles are alleles whose specificity is predicted on the basis of B and F pocket structure to overlap with the supertype specificity.
level of DNA typing, correspondence to serologically defined Ag frequencies was assumed (48). Gene frequencies were calculated from phenotypic frequencies utilizing the binomial distribution formula (48). Estimates of the redundancy or breadth of coverage afforded by a specific panel of epitopes were derived using the game theory Monte Carlo simulation methodology (49).

**HLA transgenic mice and cell lines**

The derivation and characterization of the HLA-A*0201/Kβ, HLA-A*1101/Kβ, and HLA-B*0702/Kβ transgenic mice were described previously (33, 50, 51). These animals express a chimeric class I molecule composed of the α1 and α2 domain of HLA class I and the α3 domain of murine class I Ags. The HLA-A*1101/Kβ transgenic mice were used as representative for the HLA-A3 supertype. Jurkat cells coexpressing the HLA-A*0201/Kβ gene and the env gene from HIV-1gag, or the full-length p55 gag gene from HIV-1HXB2 were generated by transfecting the pCEI expression vector expressing the HIV-1 genes into HLA-A*0201/Kβ Jurkat cells using electroporation. Transfected cells were selected by growth in 200 μM hygromycin.

**Human subjects**

HIV-1-infected study subjects were selected from a cohort of individuals followed in the Adult Infectious Diseases Group Practice at the University of Colorado Health Sciences Center (UCHSC). HIV-1-negative subjects were normal healthy adult volunteers. The study was approved by the UCHSC Institutional Review Board, and all study subjects participated voluntarily and gave informed consent. PBMC were obtained from the heparinized blood of each subject by gradient-density centrifugation and were immediately cryopreserved. A total of 53 HIV-1-infected subjects and 13 healthy, uninfected control subjects was included in this study. The HIV-1-infected subjects were divided into two clinical cohorts defined as suppressed and viremic based on viral load, which was assessed as a function of plasma HIV-1 RNA levels measured using the Roche HIV-1/1 Monitor kit (Roche Laboratories, Somerville, NJ). Subjects receiving combination antiretroviral therapy with plasma HIV-1 RNA levels of <1000 copies/ml for at least 6 mo were considered to be suppressed, whereas subjects with a plasma viral load of >1000 HIV-1 RNA copies/ml, regardless of antiretroviral treatment status, were defined as viremic.

**HLA typing**

Molecular and serologic HLA typing was performed at the UCHSC Clinical Immunology HLA laboratory from either whole blood samples or BEV-transformed B cell lines. All results were verified with low resolution PCR sequence-specific primer typing from Genovision (West Chester, PA) (52).

**Human IFN-γ ELISPOT assay**

PBMC responses to the panel of CTL epitope peptides were evaluated using an IFN-γ ELISPOT assay, as described (53). PBMC from each of these nontransduced, murine class I-negative 96-well plates (Millepore, Bedford, MA) were coated overnight at 4°C with the murine mAb specific for human IFN-γ (clone 1-D1K; Mabtech, Cincinnati, OH) at the concentration of 5 μg/ml. After washing with PBS, RPMI + 10% heat-inactivated human AB serum was added to each well and incubated at 37°C for at least 1 h to block membranes. The CTL epitope peptides were diluted in AIM-V media and added to triplicate wells in a volume of 100 μl at the final concentration of 10 μg/ml. Cryopreserved PBMC were thawed, resuspended in AIM-V at a concentration of 1 x 10^6 PBMC/ml, and dispensed in 100 μl vol into test wells. The assay plates were incubated at 37°C for 40 h, after which they were washed with PBS + 0.05% Tween 20. To each well, 100 μl of biotinylated mAb specific for human IFN-γ (clone 7-B6-1; Mabtech) at the concentration of 2 μg/ml was added and plates were incubated at 37°C for 2 h. The plates were again washed, avidin-peroxidase complex (Vectorstain Elite kit) was added to each well, and the plates were incubated at room temperature for 1 h. The plates were developed and read, as described for the murine ELISPOT.

**Statistical methods**

All analyses were completed, assuming a two-sided significance level of 0.05. Statistical analyses were conducted using SAS/STAT (SAS Institute, Cary, NC) and Splus (Insightful, Seattle, WA). Wilcoxon signed rank tests were used for between-group comparisons of continuous outcomes. χ² tests were used to determine whether subjects with a particular HLA type (HLA-A2, HLA-A3, or HLA-B7 supertypes) were more likely to respond to the corresponding epitopes than patients without the predicted HLA type. The overall probability of response to HLA-A2, HLA-A3, or HLA-B7 super-type epitopes given relevant allele expression was modeled using a logistic-normal model for analyzing binary outcome data with repeated observations. This model was programmed using SAS’S NLMIXED procedure. The final model was chosen based on Akaike’s Information Criteria.

**Design and construction of the multi-CTL epitope DNA vaccine, EP HIV-1090**

The EP HIV-1090 vaccine component was designed using computer-based modeling to optimize proteosme-mediated epitope processing and to minimize the creation of junctional epitopes, which are created by the juxtaposition of two epitopes. These properties were controlled by altering epitope order and through the introduction of selected amino acid spacers at the C terminus of individual epitopes (54). Finally, the gene product design was optimized to support preferred human codon usage.

This component of the vaccine was constructed using overlapping oligonucleotides in a PCR-based synthesis (55). Overlapping oligonucleotides, averaging 60–90 bp in length with overlaps of ~15–20 bp, were synthesized and HPLC purified by Operon Technologies (Alameda, CA). Constructs were assembled by extending the overlapping oligonucleotides using PFU polymerase (Strategene, San Diego, CA). A consensus Igs signal sequence was fused to the 5′ end of the gene product, to facilitate transport of the expressed protein into the endoplasmic reticulum. The resulting full-length product was sequenced and subcloned into a clinically acceptable plasmid vector, pMB75.6 (Valentis, Burlingame, CA). Expression of the vaccine gene is driven by the CMV-IE promoter, and the only protein that can be expressed in eukaryotic cells transfected with this DNA vaccine are the CTL and Pan DR epitope (PADRE) epitopes. Neither the pMB75.6 vector backbone nor the epitope-encoding region shares significant homology with known human genomic sequences.

**EP HIV-1090 DNA vaccine**

EP HIV-1090 DNA was produced by growth in Escherichia coli (strain DH5α) in Terrific Broth with kanamycin (25 μg/ml) and purified using Qiagen MegaPrep columns, according to the manufacturer’s directions (Qiagen, Valencia, CA). A clinical formulation of EP HIV-1-1090 was developed for phase I clinical testing. In this formulation, the DNA plasmid was mixed with polyvinylpyrrolidone (PVP; Plasdone, International Specialty Products, Wayne, NJ) at a ratio of 17 parts PVP to 1 part DNA, in PBS, pH 7.0. When used in DNA vaccine formulations, PVP binds to DNA, which facilitates uptake by skin and muscle cells. Formulations supplemented with PVP have been tested in numerous animal species, including mice, Beagle dogs, and pigs (56, 57).

**EP HIV-1090 DNA vaccine immunogenecity testing in HLA transgenic mice**

CTL responses were generally measured after a single immunization using splenic lymphocytes obtained 11–14 days following immunization. Direct assessment of epitope immunogenicity was completed using synthetic peptides, 50 μg/dose emulsified in IFA with 140 μg/dose of the hepatitis B virus core 128 helper epitope, which were administered s.c. to 6–19 HLA transgenic mice. When the EP HIV-1090 DNA vaccine was used, groups of 6–9 HLA transgenic mice were immunized bilaterally with 100 μg of DNA into tibialis anterior muscle, which was pretreated by cardiotony injection (55). Immunogenicity studies completed using the EP HIV-1090 DNA vaccine formulated with PVP were completed without the cardiotony pretreatment of muscle at the injection site and using two immunizations administered 7 days apart.

Two related assays were used to measure CTL activity induced by immunization in the HLA transgenic mice, an ELISPOT and the in situ assay ELISA (58). For both assays, the production of IFN-γ by T lymphocytes is used as the assay readout. For ELISPOT assays, purified CD8 cells (4 x 10^6/well) and irradiated splenocyte cells (10^6 cells/well) were added to membrane-based 96-well ELISA plates (Millepore, Bedford, MA) coated with anti-IFN-γ mAb (BD Pharmingen, San Diego, CA). Cells were cultured with 10 μg/ml peptide for 20 h at 37°C. The number of IFN-γ-secreting cells was detected by incubation with biotinylated anti-mouse IFN-γ Ab (BD Pharmingen), followed by incubation with avidin-peroxidase complex (Vectastain). Finally, the plates are developed using 3-aminon-9-ethyl-carbazole (Sigma-Aldrich, St. Louis, MO), washed, and dried. Spots are counted utilizing the Zeiss (Oberkochen, Germany) KS ELISPOT reader.

In the case of in situ ELISA assays, the splenocytes (2.5 x 10^7) were cultured with peptide (1 μg/ml) and irradiated LPS-activated splenocytes (10^6) in RPMI medium for 6 days at 37°C in 5% CO2. After this 6-day

*Abbreviations used in this paper: PADRE, Pan DR epitope; HAART, highly active antiretroviral therapy; PVP, polyvinylpyrrolidone; SPC, spot-forming cell; SU, secretory unit.*
restimulation, serially diluted splenocytes were cultured for 20 h with and without peptide (1 μg/ml) and 10^5 Jurkat A2.1/Kb in ELISA plates (Costar, Corning, NY) coated with rat mAb-specific murine IFN-γ (clone RA-6A2; BD PharMingen). The following day, the cells were removed by washing the plates with PBS with Tween 20, and the amount of IFN-γ that was secreted and captured by the bound clone RA-6A2 mAb was measured using a sandwich format ELISA. In situ IFN-γ ELISA data were converted to secretory units (SU) for evaluation (58).

Results

Identification of potential HIV-1 vaccine candidate CTL epitopes

The initial efforts to scan amino acid sequences of Env, Gag, Pol, Nef, Rev, Tat, Vif, Vpr, and Vpu for the presence of amino acid motifs known to be characteristic for peptides capable of binding to HLA-A2, HLA-A3, or HLA-B7 supertype alleles yielded

<table>
<thead>
<tr>
<th>Table II. HLA supertype binding</th>
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<tbody>
<tr>
<td>Epitope</td>
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<tr>
<td>---------</td>
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<tr>
<td>A2 Supertype</td>
</tr>
<tr>
<td>Gag 386</td>
</tr>
<tr>
<td>Nef 221</td>
</tr>
<tr>
<td>Pol 448</td>
</tr>
<tr>
<td>Env 134</td>
</tr>
<tr>
<td>Pol 498</td>
</tr>
<tr>
<td>Vpr 62</td>
</tr>
<tr>
<td>Gag 271</td>
</tr>
</tbody>
</table>

| A3 Supertype |
| Pol 929 | QMVFIFHNFK | 9.2 | 8.5 | 269 | 433 | 400 |
| Pol 971 | KIQNFLYVR | 344 | 28.6 | 2.7 | 341 | 211 |
| Env 47 | VTVYYGVFK | 84.6 | 11.3 | 4615 | — | 170 |
| Pol 98 | VTIKGQQLK | 297 | 28.6 | — | — | 125 |
| Pol 347 | AIFQSMTP | 10.0 | 10.0 | — | — | 242 |
| Pol 722 | KTVLAWFPAK | 3.5 | 7.6 | 164 | 3580 | 8000 |
| Env 61 | TTLFCASDAK | 120 | 27.3 | 9474 | — | 140 |

| B7 Supertype |
| Nef 94 | FPVRPQVPL | 15.7 | 43.0 | 11.8 | 482 | 71.4 |
| Pol 454 | YPLALRSLF | 393 | 480 | 39.3 | 150 | 714 |
| Env 259 | IFIYHCAPA | 423 | 343 | 163 | — | 3.7 |
| Rev 75 | VPQLQPQL | 112 | 6000 | 0.8 | — | 270 |
| Gag 237 | HPVHAGPIA | 50.0 | 11.6 | — | 4429 | 4.3 |
| Pol 893 | IPYNPQSQGQ | 458 | — | 120 | — | 66.7 |
| Env 250 | CPKVSFEPI | 100 | 5143 | 162 | 2447 | 100 |

<table>
<thead>
<tr>
<th>Table III. Conservation of vaccine candidate CTL epitopes across subtypes</th>
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<tr>
<td>Epitope</td>
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<tr>
<td>Gag 386</td>
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<tr>
<td>Nef 221</td>
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<tr>
<td>Pol 448</td>
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<td>Rev 75</td>
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<tr>
<td>Gag 237</td>
</tr>
<tr>
<td>Pol 893</td>
</tr>
<tr>
<td>Env 250</td>
</tr>
<tr>
<td>Average</td>
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</table>

^a Total conservation based on analysis of 64 sequences from subtypes A, AC, AE, AG, B, C, D, F, G, H, J, N, and O.
^b Conservation in subtype A isolates based on analysis of 4 sequences.
^c Conservation in subtype B isolates based on analysis of 19 sequences.
^d Conservation in subtype C isolates based on analysis of 8 sequences.
^e Conservation in subtype D isolates based on analysis of 4 sequences.
>50,000 potential CTL epitopes. A subset of these motif-bearing sequences was selected for further study on the basis of conservancy primarily among subtype B viral variants. Sequence-conserved peptides represented ~2% of the total motif-bearing peptides. For example, for the HLA-A2 epitope identification, >20,000 motif-positive peptides were identified and 233 conserved peptides were subsequently synthesized to analyze binding to purified HLA class I molecules. Through this screening process, a set of 48 candidate CTL epitopes was identified and a panel of 21 epitopes was selected for use in the vaccine, seven epitopes for each of the selected HLA supertypes, based on binding to multiple supertype alleles (Table II). Each of these selected epitopes bound with high affinity to at least three HLA alleles within each HLA supertype.

Conservation and predicted population coverage of the HLA-A2, HLA-A3, and HLA-B7 supertype CTL epitopes

The CTL epitopes identified are intended to form the basis of a HIV-1 vaccine for global application, and as such, the representation of these epitopes in nonsubtype B HIV-1 isolates and potential population coverage were also examined. As shown in Table III, some of the 21 supertype epitopes are present intact in greater than 90% of the 64 HIV-1 isolates tested; this includes sequences from subtype A, C, and D isolates. On average, the epitopes were conserved in 58% of the viral isolates analyzed, and at least one epitope from each of the respective HLA supertypes was conserved in greater than 90% of the sequences. Epitopes were similarly conserved when analyzed on an individual subtype basis; overall conservation of these epitopes in subtypes A, B, C, and D was 52, 78, 53, and 64%, respectively. Although certain epitopes, such as Gag 271, Pol 722, and Gag 545, were poorly conserved, the majority of epitopes were more frequently found in HIV-1 isolates; for some of the subtypes, the epitopes were conserved in 100% of the sequences examined.

Based on reported allelic frequencies, ~85% of randomly selected individuals would be predicted to be genetically capable of producing CTL responses to one or more of the selected epitopes (Table IV). On average, an individual would be predicted to recognize 8.1 individual CTL epitopes. Similar estimates are obtained when predicted coverage was calculated within defined ethnic populations (Table IV). These data suggest that a vaccine composed of these 21 CTL epitopes would be relevant to many, if not most, of the populations in the world.

Immunological recognition of vaccine candidate epitopes by PBMC from HIV-1-infected subjects

The relative antigenicity of each of the 21 HLA-A2, HLA-A3, and HLA-B7 supertype-restricted epitopes identified during the course of the binding studies was evaluated in a cohort of 53 HIV-1-infected subjects utilizing recall IFN-γ ELISPOT assays. The ELISPOT responses to individual peptides in PBMC from all HIV-1-infected donors vs seronegative controls are depicted in A and B, respectively. Based on the results in seronegative donors, a significantly positive peptide response was considered to be greater or equal to 5 net SFC per 10⁵ PBMC plated. Using this criterion, all the epitopes tested, except for the HLA-B7-restricted Pol 893, were recognized by CTL from at least one HIV-1-infected subject. Of the total HIV-infected patient cohort tested, 70% of subjects recognized 1 or more of the epitopes, and 49% recognized 2 or more. The median number of epitopes recognized per subject in the total group was 1, with a range of 0–10 epitopes.

The magnitude and breadth of peptide-specific CTL responses were compared between 29 HIV-infected subjects effectively

Table IV. Estimated population coverage of the vaccine CTL epitopes in different ethnic populations

<table>
<thead>
<tr>
<th></th>
<th>Asian</th>
<th>Black</th>
<th>North American Caucasians</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Population coverage</td>
<td>84.4</td>
<td>78.0</td>
<td>88.9</td>
<td>85.1</td>
</tr>
<tr>
<td>Average no. epitopes</td>
<td>7.4</td>
<td>5.9</td>
<td>9.4</td>
<td>8.1</td>
</tr>
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treated with highly active antiretroviral therapy (HAART), denoted as suppressed, and 21 subjects with active HIV-1 plasma viremia (viremic, Fig. 2, A and B). Summed ELISPOT responses to the 21 peptides were greater in viremic subjects than in those suppressed on HAART \( (p = 0.0516, \text{Fig. 3A}) \), as might be expected based on reports of declining CTL responses on effective therapy \( (59) \). The breadth of epitope recognition was also somewhat greater in viremic subjects, with a median of two epitopes recognized per HIV-1-infected subject compared with a single epitope recognized in the antiretroviral suppressed individuals \( (p = 0.286) \).

Demonstration that the selected CTL epitope peptides can be recognized by CD8\(^+\) T lymphocytes from HIV-1-infected subjects was considered important because this confirms that each epitope is actually processed from viral gene products during the course of natural HIV-1 infection. These data demonstrate that the majority of the selected CTL epitopes tested were recognized by HIV-1-infected subjects, suggesting that they represent natural epitopes. However, despite epitope antigenicity, CTL recognition was typically narrowly directed toward only a few epitopes, regardless of the level of plasma viremia.

**Influence of HLA type on immune recognition of supertype epitopes**

One important consideration of a vaccine based on HLA supertype epitopes is its predicted population coverage. Since a significant percentage of the HIV-infected individuals tested recognized one or more of the epitope peptides, we next sought to determine the relationship between epitope recognition and HLA supertype expression. To determine whether observed ELISPOT responses to the supertype peptides were predicted by HLA class I type, HLA-typed, HIV-1-infected subjects were grouped according to their expression of HLA alleles, those both verified and predicted according to Table I, as falling into either the HLA-A2, HLA-A3, or HLA-B7 superfamilies. Of the 39 HLA-typed subjects evaluated, 37 expressed one or more alleles within a superfamily, with 20 expressing HLA-A2, 22 expressing HLA-A3, and 16 expressing HLA-B7 supertype alleles. Of all the subjects expressing one or more supertype alleles, 73\% recognized a peptide restricted by their allele, as predicted by HLA-peptide-binding studies (data not shown). The probabilities of response to one or more epitopes within a subject’s HLA supertype were 0.70 (14 of 20), 0.59 (13 of 22), and 0.63 (10 of 16) for the HLA-A2, HLA-A3, and HLA-B7 superfamilies, respectively. These response probabilities were lower in subjects without the corresponding HLA type. The probability of measuring CTL responses specific for epitopes outside of HLA types was greater for the HLA-A2 and HLA-A3 epitopes, with response probabilities of 0.42 (8/19) \( (p = 0.0791) \) and 0.47 (8/17) \( (p = 0.4548) \), respectively, whereas the probability for unpredictable responses to the HLA-B7 epitope was only 0.13 (3 of 23) \( (p = 0.0013) \). Using a logistic-normal model for analyzing binary outcome data with repeated observations, the odds in favor of a response to predicted HLA-A2, HLA-A3, or HLA-B7 CTL epitopes were estimated to be 5.7 (95\% confidence interval 1.9, 16.6) times the odds in favor of a response for subjects without relevant HLA type, a highly significant difference \( (p = 0.0015) \). These data indicate that the process used for identifying the CTL epitopes was highly predictive.

PBMC ELISPOT responses to each epitope peptide restricted by a given supertype in subjects expressing a relevant supertype allele were assessed for magnitude and breadth of response to identify potential dominance relationships (Fig. 4). Significant CTL responses were measured using PBMC from individuals in all three of the selected HLA supertype groups. In this cohort of HIV-1-infected subjects, positive ELISPOT responses associated with the
HLA-A3 phenotype were generally of the greatest magnitude and were evenly directed against the set of selected A3 supertype peptides (Fig. 4B). In contrast, ELISPOT responses measured in HLA-A2 and HLA-B7 subjects were often of lower magnitude and focused on a more limited number of epitopes (Fig. 4, A and C).

Immunogenicity of the EP HIV-1090 DNA vaccine in HLA transgenic mice

The plasmid-based vaccine, EP HIV-1090, was designed to express the 21 vaccine candidate CTL epitopes and the universal HTL epitope, PADRE, as a single gene product (Fig. 5). The immunogenicity of the EP HIV-1090 DNA vaccine was characterized using HLA transgenic mice. Immune responses induced using peptide immunizations were used for comparison, to assess relative potency of the DNA vaccine. The results of the immunogenicity studies in the HLA-A2, HLA-A11, and HLA-B7 transgenic mice obtained using the in situ ELISA are shown in Fig. 6A. The HLA-A3-restricted epitope, Env 61, is not shown, as this epitope is not immunogenic in the transgenic mice. Experimental vaccination with 100 µg of the EP HIV-1090 DNA vaccine induced HLA-A2- and HLA-A3-restricted CTL responses that were generally equal or of greater magnitude than the responses induced by peptide immunization. Although the HLA-B7 transgenic animals are typically less responsive to both peptide and DNA immunization, three of the HLA-B7 epitopes in the EP HIV-1090 DNA vaccine induced measurable responses. The immunogenicity of the EP HIV-1090 vaccine was further characterized in HLA-A2 and HLA-A11 transgenic mice using the IFN-γ ELISPOT assay, in which the frequencies of epitope-specific CTL were measured in the absence of in vitro peptide restimulation. The seven HLA-A2-restricted epitopes and five of six of the HLA-A3-restricted epitopes induced significant CTL responses (Fig. 6B). The magnitude of the responses, in general, exceeded 100 SFC/10^6 CD8^+ T lymphocytes.

Assays completed to initially assess the DNA vaccine immunogenicity were based on the use of peptide-loaded target cells, and as such, the data do not demonstrate the ability of the vaccine-induced CTL to effectively recognize cells endogenously expressing intact HIV-1 gene products. To address this issue, Jurkat cells coexpressing the HLA-A*0201/Kb gene and the Env gene from HIV-1 JR-FL or the Gag gene from HIV-1 HXB2 were used as CTL target cells. Vaccine-induced CTL were evaluated using the in situ ELISA following in vitro restimulation with one of three representative HLA-A2-restricted epitopes: Env 134, Gag 271, and Gag 386. The responding CTL recognized the whole gene transfected target cells comparably to peptide-loaded target cells (Fig. 7). These results demonstrate that vaccine-induced CTL are capable of recognizing cells expressing intact HIV-1 genes.

Immunogenicity testing of the clinical formulation of EP HIV-1090

The CTL responses induced in individual HLA-A2 transgenic mice immunized with EP HIV-1090 DNA vaccine formulated with PVP were measured using the ELISPOT assay. Responses by CTL obtained from individual mice were measured to directly assess the ability of the vaccine to induce responses specific to different epitopes in the same individual, the situation analogous to the desired outcome in clinical testing. Due to limited number of cells available from a single mouse, splenocytes were restimulated in vitro with a pool of the HLA-A2-restricted epitope peptides, and individual responses were measured 6 days later. Significant CTL responses to all epitopes were detected in most mice, and none of the epitopes was clearly immunodominant (Fig. 8). Thus, the EP HIV-1090 DNA vaccine can be used to induce CTL responses with significant breadth in individual animals.

Discussion

Although the public health need for a vaccine against HIV-1 is well recognized and accepted, there exist numerous obstacles to
the design and development of a vaccine for global application. Vaccine design strategies to address the genetic variation of HIV-1 isolates are one of the most significant obstacles. Some proposed strategies base vaccines specifically on HIV-1 types prevalent within specific populations or use ancestral or consensus sequences based on HIV-1 types in the local target population. However, these approaches are themselves associated with potential limitations. Most significant is the possibility that viral evolution, potentially driven by vaccine-induced immune responses, may quickly limit the utility of any HIV-1 type-specific vaccine. Viral escape from CTL responses, induced as the result of both natural infection and vaccines, has documented in nonhuman primate models with the mechanism behind the escape was mutation of the anchor residues in dominant CTL epitopes. The logic behind this approach is that conserved regions of the viral genome are those that have been maintained through the evolutionary process, a set of 48 conserved HIV-1 CTL epitopes restricted by HLA-A2, HLA-A3, and HLA-B7 alleles was identified for initial vaccine development efforts, of which 21 were selected for further study.

Antigenicity data from HIV-1-infected subjects are particularly informative, as they confirm that each peptide is likely to be processed from the respective viral Ag during the course of natural HIV-1 infection. Previous studies had shown that each of the HLA-A2 and HLA-A3 supertype epitopes was recognized in recall CTL assays using lymphocytes from HIV-infected subjects. Three of the seven HLA-B7 supertype-restricted peptides, Nef 94 (75), Env 250, and Env 259 (76), were also previously reported to be natural epitopes recognized in HIV-1-infected subjects. However, the combination of supertype epitopes contained in the EP-1090 vaccine had never been tested in the same cohort of infected subjects, nor had the impact of HLA type on CTL recognition been thoroughly investigated. Thus, the demonstration of CTL epitope recognition, utilizing PBMC obtained from HIV-1-infected individuals, could be used to effectively validate the epitope selection processes. Although the primary goal driving these studies was to establish antigenicity of the epitopes, additional interesting immune response data relating to the frequency and the HLA restriction of the epitope-specific responses were also generated. First, while 20 of the vaccine epitopes were recognized by CTL from at least one HIV-1-infected donor, the measured CTL responses in a given HIV-1-infected individual were typically narrowly directed against only a single or few epitopes. This observation is in contrast with several recent reports in which very broad CTL responses were detected during both the acute and chronic stages of infection. These other studies were completed using naturally infected volunteers and immune assays with overlapping sets of peptides to identify CTL epitopes, whereas we utilized epitope prediction based on binding motifs, high affinity supertype HIV-1 peptide binding, and viral conservation data as the primary criteria. Thus, these different findings might be attributed to the different processes used to identify the CTL epitopes, and the limited frequency of CTL recognition for the selected vaccine epitopes in natural infection would not reduce their potential utility in vaccines. In fact, one interpretation of these results is that vaccine approaches specifically designed to target multiple, highly conserved epitopes have the potential to...
generate more effective T cell responses than those induced during natural infection.

Analysis of HLA phenotypes in conjunction with the occurrence of CTL responses to specific epitopes revealed that the pattern of epitope restriction was highly predictable and statistically significant. The epitope motif analysis and HLA-peptide-binding data provided a means to correctly predict genetic restriction of the CTL responses. However, additional responses were observed in which the HLA type of the HIV-1-infected volunteer was different than what was assigned to the epitope based on HLA-peptide epitope binding. The simplest explanation for the expanded rate of immune recognition is that some of these epitopes contain motifs capable of mediating binding to HLA alleles outside of the defined supertype families. For example, the Nef 221 epitope (LTFGWCFL; anchor residues are shown bolded), which was selected as a HLA-A2 epitope, contains a nested HLA-A3-binding motif. Truncated forms of this peptide, bearing a lysine at the C terminus, readily bind HLA-A3 superfamily alleles (LTFGWCFK; anchor residues are shown bolded). The binding motifs for HLA-A2 and HLA-A3 epitopes share some of the same amino acids (Table I), so binding of an individual epitope peptide to both HLA products, as well as recognition of this single epitope by CD8+ T lymphocytes from HLA-A2- and HLA-A3-positive individuals, is expected.

This observation may explain, in part, the breadth of CTL responses noted for the HLA-A2 and HLA-A3 epitopes, compared with the HLA-B7-restricted epitopes; the HLA-B7-binding motif is more unique. Alternatively, these epitopes may bind to other HLA allelic products that belong to other, less defined supertypes or HLA-A or HLA-B molecules or even HLA molecules encoded by different genes, such as HLA-C. Further analyses and continued definition of the HLA supertypes are ongoing efforts.

FIGURE 6. Immunogenicity of EP HIV-1090. A, HLA transgenic mice were immunized bilaterally in cardiotoxin-treated tibialis anterior muscle with 100 µg of EP HIV-1-1090 or s.c. with 50 µg of individual peptide emulsified in IFA. Eleven days after immunization, spleens from each experimental group were pooled, and isolated splenocytes were restimulated in vitro with peptide-loaded APC for 6 days. To more conveniently compile results from different experiments, the level of CTL activity, measured as a function of IFN-γ secretion, was expressed as SU. CTL responses in HLA-B7 transgenic mice were measured after two in vitro restimulations. B, CD8+ T cells were purified from splenocytes of EP HIV-1090-immunized animals or unimmunized control animals. The frequency of epitope-specific CTL responses was measured in the absence of peptide re-stimulation using an IFN-γ ELISPOT assay.
It should be noted that recognition for 20 of the 21 epitopes in the vaccine was demonstrated using PBMC from this small cohort of HIV-infected volunteers. The unanticipated CTL recognition of epitopes outside of the predicted HLA supertype represents additional responses, and the occurrence of these does not reduce utility of the epitope identification process used or the potential value of these epitopes for vaccine use. In reality, this increased rate of epitope recognition may increase the utility of the vaccine by increasing the numbers of epitopes an individual is capable of responding against, and this would increase vaccine efficacy.

A commonly cited concern associated with the use of defined epitopes to produce vaccines is that selected epitopes may not be optimally immunogenic. Poor immunogenicity can be a legitimate concern when epitopes are identified using only computer-based motif scans because many motif-positive peptides will not bind to HLA molecules with high affinity. The affinity of HLA-peptide binding has proven to be highly predictive of immunogenicity for several viral pathogens (33, 80, 81), including HIV-1 (53). Thus, HLA-peptide-binding measurements are considered a critical component of the overall process.

Restricted population coverage has also been identified as a potential limitation to the use of epitopes-based vaccines. This can be problematic when allele-specific epitopes are identified, but our focus on HLA supertypes is meant to overcome this potential limitation (23, 48, 82). To directly test this theory, we analyzed the HLA-binding affinity of the vaccine epitopes with respect to known HLA phenotype frequencies in different ethnic groups. Based on this analysis, greater than 85% of the general population will express an HLA allele capable of binding at least two of these epitopes, and on average individuals would be predicted to be capable of binding more than eight epitopes. While these estimates are significantly higher than the frequency of immune recognition observed in the HIV-1-infected subjects, such predictions are reasonable given the immunochemical characteristics of the epitopes and that the vaccine, unlike the virus itself, is designed to stimulate the induction of CTL responses specifically against these epitopes.

The sole reliance on immune response data, obtained using PBMC from HIV-1-infected individuals, can also lead to the identification of CTL epitopes with limited utility for vaccines because of epitope sequence variation among viral types and allele-specific restriction. For example, the majority of 15 HLA-A3- and 11 HLA-B7-restricted CTL epitopes identified as immunogenic in a cohort of acutely infected HIV-1 patients using a set of overlapping synthetic peptides (79) were previously identified using the motif-based identification processes described in the present study. However, only one of the HLA-B7-restricted epitopes and six of the HLA-A3-restricted epitopes found by Yu and colleagues were conserved and bound with moderate to high affinity to more than a single HLA-A3 or HLA-B7 supertype allelic product. Although these epitopes are clearly immunogenic and recognized during natural infection, their use in a vaccine would not likely provide the desired levels of viral strain coverage or population coverage.

EP HIV-1090 is a DNA plasmid-based vaccine encoding 21 conserved HIV-1 CTL epitopes restricted by HLA-A2, HLA-A3, and HLA-B7 supertype alleles and the universal HTL epitope, PA-DRE. The epitopes are arranged in a single open reading frame and separated by one to four amino acid spacers, a vaccine design feature incorporated to optimize proteosome processing and, subsequently, epitope and vaccine immunogenicity (54). The potency of EP HIV-1090 DNA vaccine was characterized in immunogenicity studies utilizing HLA transgenic mice, in which the vaccine was demonstrated to induce CTL responses to multiple epitopes.

One component of our working hypothesis is that the induction of a broadly reactive CTL response within an individual vaccine recipient will be beneficial. This assumption is based on studies of long-term nonprogressors, in which broadly reactive CTL responses were observed (3–11, 83, 84). The question of competition or immunological dominance between CTL epitopes, which would effectively reduce the breadth of the total response induced by vaccination, was raised in two recent publications (85, 86). The factors that are likely to contribute to immunodominance need to be accounted for in the design of vaccines, in which the prescribed goal is the induction of CTL responses against multiple epitopes. MHC-binding affinity, efficiency of epitope processing, and competition between T cells for access to APCs can all contribute to establishing an immunodominant response (87). The selection of
epitopes with similar high affinity MHC binding and the optimization of epitope processing are inherent to the design of EP HIV-1090. To directly address this issue, we immunized HLA-A2 transgenic mice with the EP HIV-1090 vaccine and measured CTL responses using splenocytes from single animals. Significant CTL responses to six to seven of the CTL epitopes encoded in the vaccine were reproducibly induced. Thus, we believe that epitope competition or detrimental dominance relationships have been controlled through our vaccine design features.

Clinical trials completed with DNA vaccines for HIV-1, which have been tested in both HIV-1-infected and uninfected volunteers, have yielded data that document their safety, but immunogenicity has been limited and generally disappointing (88–93). However, the potential for priming cellular immune responses with multiple DNA immunizations, with or without the need for viral vector boosts, supports their continued development. The EP HIV-1090 DNA vaccine incorporates numerous design properties that clearly boost, support their continued development. The EP HIV-1090 vaccine incorporates numerous design properties that clearly boost, support their continued development. The EP HIV-1090 vaccine incorporates numerous design properties that clearly boost, support their continued development.

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