Utilization of MHC Class I Transgenic Mice for Development of Minigene DNA Vaccines Encoding Multiple HLA-Restricted CTL Epitopes

Glenn Y. Ishioka, John Fikes, Gary Hermanson, Brian Livingston, Claire Crimi, Mingsheng Qin, Marie- France del Guercio, Carla Oseroff, Carol Dahlberg, Jeff Alexander, Robert W. Chesnut and Alessandro Sette

*J Immunol* 1999; 162:3915-3925; ;
http://www.jimmunol.org/content/162/7/3915

**Why The JI?**

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article cites 41 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/162/7/3915.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852 Copyright © 1999 by The American Association of Immunologists All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Utilization of MHC Class I Transgenic Mice for Development of Minigene DNA Vaccines Encoding Multiple HLA-Restricted CTL Epitopes

Glenn Y. Ishioka, John Fikes, Gary Hermanson, Brian Livingston, Claire Crimi, Mingsheng Qin, Marie-France del Guercio, Carla Oseroff, Carol Dahlberg, Jeff Alexander, Robert W. Chesnut, and Alessandro Sette

We engineered a multiepitope DNA minigene encoding nine dominant HLA-A2.1- and A11-restricted epitopes from the polymerase, envelope, and core proteins of hepatitis B virus and HIV, together with the PADRE (pan-DR epitope) universal Th cell epitope and an endoplasmic reticulum-translocating signal sequence. Immunization of HLA transgenic mice with this construct resulted in: 1) simultaneous CTL induction against all nine CTL epitopes despite their varying MHC binding affinities; 2) CTL responses that were equivalent in magnitude to those induced against a lipopeptide known to be immunogenic in humans; 3) induction of memory CTLs up to 4 mo after a single DNA injection; 4) higher epitope-specific CTL responses than immunization with DNA encoding whole protein; and 5) a correlation between the immunogenicity of DNA-encoded epitopes in vivo and the in vitro responses of specific CTL lines against minigene DNA-transfected target cells. Examination of potential variables in minigene construct design revealed that removal of the PADRE Th cell epitope or the signal sequence, and changing the position of selected epitopes, affected the magnitude and frequency of CTL responses. Our results demonstrate the simultaneous induction of broad CTL responses in vivo against multiple dominant HLA-restricted epitopes using a minigene DNA vaccine and underline the utility of HLA transgenic mice in development and optimization of vaccine constructs for human use. The Journal of Immunology, 1999, 162: 3915–3925.

Several studies point to the crucial role of cytotoxic T cells in the eradication of infectious diseases and cancer by the immune system (1–3). Recombinant protein vaccines do not reliably induce CTL responses, and the use of otherwise immunogenic vaccines consisting of attenuated pathogens in humans is hampered, in the case of several important diseases, by overriding safety concerns. Furthermore, for diseases such as HIV, hepatitis B virus (HBV), hepatitis C virus (HCV), and malaria, it appears important to not only induce a vigorous CTL response, but to also focus the CTL response against highly conserved epitopes to prevent escape by mutation.

Induction of a broad response directed simultaneously against multiple epitopes also appears to be crucial for the development of efficacious vaccines against several important diseases. HIV infection is perhaps the best example where an infected host may benefit from a multispecific response. Rapid progression of HIV infection has been reported in cases where a narrowly focused CTL response is induced, whereas nonprogressors tend to show a broader specificity of CTLs (4, 5). Also, evidence showing great variability of many HIV CTL epitopes resulting from the error prone replication of the RNA genome and selection of escape mutants by CTL responses directed against only a single or few epitopes also supports the need for broad epitope CTL responses (6).

One potential approach to induce multispecific responses against conserved epitopes is genetic immunization with a minigene encoding the epitopes in a “string-of-beads” fashion. Induction of CTL, Th, and B cell responses, restricted by murine MHC molecules, have been described by several laboratories using constructs encoding as many as 11 epitopes (7–10). Minigenes have been delivered in vivo by infection with recombinant adenovirus or vaccinia or by injection of purified DNA via the i.m. or intradermal route (11, 12).

Successful development of minigene DNA vaccines for human use will require addressing certain fundamental questions related to epitope MHC affinity, optimization of constructs for maximum in vivo immunogenicity, and development of assays for testing in vivo potency of multiepitope minigene constructs. Regarding MHC binding affinity of epitopes, it is not currently known whether both high- and low-affinity epitopes can be included within a single minigene construct, and what ranges of peptide affinity are permissible for CTL induction in vivo. This is especially important because dominant epitopes can vary in their affinity and because it might be important to be able to deliver mixtures of dominant and subdominant epitopes that are characterized by high and low MHC binding affinities.

With respect to minigene construct optimization for maximum immunogenicity in vivo, conflicting data exists regarding whether the exact position of the epitopes in a given construct or the presence of flanking regions, Th cell epitopes, and endoplasmic...
reticulum (ER)-translocating signal sequences might be crucial for CTL induction (13–20).

Regarding development of assays that allow testing of human vaccine candidates, it would be advantageous to be able to test the in vivo immunogenicity of minigenes containing human CTL epitopes in a convenient animal model system. One system that could provide a useful model of CTL induction in humans is HLA-expressing transgenic mice, where previous studies demonstrated the similarity between CTL repertoires in HLA transgenic mice and humans (21, 22).

In the current study, we examine the immunogenicity of a simplified minigene construct that encodes six HLA A2.1- and three A11-restricted CTL epitopes and the universal Th cell epitope PADRE (23). We sought to determine whether a balanced and broad CTL response could be obtained in vivo against a collection of dominant epitopes deliberately selected to cover a wide range of MHC binding affinities. We also wished to compare the magnitude of the CTL responses following minigene DNA immunization to that of the Theradigm-HBV lipopeptide, a vaccine construct that has been shown to be highly immunogenic in humans (24). Finally, variables that may be potentially critical for in vivo immunogenicity, such as the presence of the PADRE T cell epitope and ER-targeting signal sequence and position of the epitopes in the construct, were also analyzed.

Materials and Methods

Peptides and lipopeptide

Peptides were synthesized according to standard F-moc solid phase synthesis methods, which have been previously described (25, 26). Peptide purity was determined by analytical reverse-phase HPLC, and purity was routinely >95%. Synthesis and purification of the Theradigm-HBV lipopeptide vaccine has been described previously (24).

Mice

HLA-A2.1/Kb transgenic mice used in this study were the F1 generation derived by crossing transgenic mice expressing a chimeric gene consisting of the α1, α2 domains of HLA-A2.1 and α3 domain of H-2Kb (27) with SJL/J mice (Jackson Laboratory, Bar Harbor, ME). This strain will be from an early generation DNA plasmid designated as pMin.0. The epitopes plasmids

Construction of multiepitope minigene and HBV Pol DNA

plasmids

The pMin.1 minigene DNA plasmid used in our studies was constructed from an early generation DNA plasmid designated as pMin.0. The epitopes and their position in the pMin.0 construct is identical to that of pMin.1 shown in Fig. 1A, with the exception that pMin.0 encodes an additional epitope, OVA 257–263, and the HBV Pol 551 epitope contains the native A residue at position 9. The position of the CTL epitopes in the pMin.0 construct was selected to minimize functional HLA-A2.1, HLA-A11, H-2Kb, and H-2Dd epitopes. In an effort to develop a more human-relevant minigene plasmid, pMin.0 was modified by substituting the HBV Pol 551 epitope with a more immunogenic analogue and deleting the OVA epitope. The resulting construct, pMin.1, showed improvements in overall immunogenicity, thus warranting its further characterization (data not shown).

Peptides and lipopeptide

Materials and Methods

Construction of multiepitope minigene and HBV Pol DNA plasmids

The pMin.1 minigene DNA plasmid used in our studies was constructed from an early generation DNA plasmid designated as pMin.0. The epitopes and their position in the pMin.0 construct is identical to that of pMin.1 shown in Fig. 1A, with the exception that pMin.0 encodes an additional epitope, OVA 257–263, and the HBV Pol 551 epitope contains the native A residue at position 9. The position of the CTL epitopes in the pMin.0 construct was selected to minimize functional HLA-A2.1, HLA-A11, H-2Kb, and H-2Dd epitopes. In an effort to develop a more human-relevant minigene plasmid, pMin.0 was modified by substituting the HBV Pol 551 epitope with a more immunogenic analogue and deleting the OVA epitope. The resulting construct, pMin.1, showed improvements in overall immunogenicity, thus warranting its further characterization (data not shown).

In the current study, we examined the immunogenicity of a simplified minigene construct that encodes six HLA A2.1- and three A11-restricted CTL epitopes and the universal Th cell epitope PADRE (23). We sought to determine whether a balanced and broad CTL response could be obtained in vivo against a collection of dominant epitopes deliberately selected to cover a wide range of MHC binding affinities. We also wished to compare the magnitude of the CTL responses following minigene DNA immunization to that of the Theradigm-HBV lipopeptide, a vaccine construct that has been shown to be highly immunogenic in humans (24). Finally, variables that may be potentially critical for in vivo immunogenicity, such as the presence of the PADRE T cell epitope and ER-targeting signal sequence and position of the epitopes in the construct, were also analyzed.

**Materials and Methods**

**Peptides and lipopeptide**

Peptides were synthesized according to standard F-moc solid phase synthesis methods, which have been previously described (25, 26). Peptide purity was determined by analytical reverse-phase HPLC, and purity was routinely >95%. Synthesis and purification of the Theradigm-HBV lipopeptide vaccine has been described previously (24).

**Mice**

HLA-A2.1/Kb transgenic mice used in this study were the F1 generation derived by crossing transgenic mice expressing a chimeric gene consisting of the α1, α2 domains of HLA-A2.1 and α3 domain of H-2Kb (27) with SJL/J mice (Jackson Laboratory, Bar Harbor, ME). This strain will be referred to hereafter as HLA-A2.1/Kb-H-2Kb+. HLA-A11/Kb transgenic mice were derived as previously described (22).

**MHC purification and peptide binding assay**

Methods for purifying HLA-A2.1 and -A11 Ag and measuring the quantitative binding of peptides to both MHC molecules have been described previously (22, 25, 26). Binding of test peptides to both MHC molecules was measured based on their capacity to inhibit the binding of a radiolabeled standard peptide. The percentage of MHC-bound radioactivity was determined by gel filtration and the concentration of the test peptide that inhibited 50% of the binding of the labeled peptide (IC50) was calculated. The standard peptides used were the HBV Core 18 peptide for A2.1 and the HIV nef 84–92 peptide for A11 (sequence AVDLYHFLK).

**Construction of multiepitope minigene and HBV Pol DNA plasmids**

The pMin.1 minigene DNA plasmid used in our studies was constructed from an early generation DNA plasmid designated as pMin.0. The epitopes and their position in the pMin.0 construct is identical to that of pMin.1 shown in Fig. 1A, with the exception that pMin.0 encodes an additional epitope, OVA 257–263, and the HBV Pol 551 epitope contains the native A residue at position 9. The position of the CTL epitopes in the pMin.0 construct was selected to minimize functional HLA-A2.1, HLA-A11, H-2Kb, and H-2Dd epitopes. In an effort to develop a more human-relevant minigene plasmid, pMin.0 was modified by substituting the HBV Pol 551 epitope with a more immunogenic analogue and deleting the OVA epitope. The resulting construct, pMin.1, showed improvements in overall immunogenicity, thus warranting its further characterization (data not shown).

**Immunization of mice**

For DNA immunization, mice were pretreated by injecting 50 μl of 10 μM cardiotxin (C9759; Sigma, St. Louis, MO) bilaterally into the tibialis anterior muscle. Four or five days later, the same muscle received 100 μg of DNA formulated in PBS.

Theradigm-HBV lipopeptide (10 mg/ml in DMSO) that was stored at −20°C was thawed for 10 min at 45°C before being diluted 1:10 (v/v) with room temperature PBS. Immediately upon addition of PBS, the lipopeptide suspension was vortexed vigorously and 100 μl was injected s.c. at the tail base (100 μg/mouse).

Immunogenicity of individual CTL epitopes was tested by mixing each CTL epitope (50 μg/mouse) with the HBV Core 128–140 peptide (TP PAYRPNAPLT, 140 μg/mouse), which served to induce IA2-restricted Th cells. The peptide mixture was then emulsified in IFA (Sigma), and 100 μl of peptide emulsion was injected s.c. at the tail base.

Cell lines and transfection

Target cells for peptide-specific cytotoxicity assays were Jurkat cells transfected with the HLA-A2.1/Kb chimeric gene (27) and 3A4-721.221 tumor cells transfected with HLA-A11/Kb (22). The parent of the latter cell line is an EBV-transformed cell line that was mutagenized and selected for loss of HLA class I expression (29). All cell lines were grown in culture medium (CM) that consisted of RPMI 1640 medium with HEPES (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 4 mM l-glutamine, 5 × 10−5 M 2-ME, 0.5 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 U/ml penicillin.

To measure presentation of endogenously processed epitopes, Jurkat-A2.1/Kb cells were transfected with pMin.1 or with a DNA construct containing the fusion of the pMin.0 and green fluorescent protein (GFP) genes, then tested in a cytotoxicity assay against epitope-specific CTL lines. The pMin.0-GFP fusion plasmid was constructed by subcloning the open reading frame of the signal sequence-deleted pMin.0 construct into pEGFP-N1 (Clontech, Palo Alto, CA) to create a N-terminal fusion with GFP. For transfection, pMin.1 and the pMin.0-GFP fusion plasmid were first subcloned into pcDNA3.1/Hygro (Invitrogen). A total of 30 μg of DNA was added to 600 μl of Jurkat-A2.1/Kb cells at 107 cells/ml, and cells were electroporated in a 0.4-cm cuvette at 0.25 kV, 960 μF. Cells were incubated on ice for 10 min before culturing for 2 days in CM. Stable transfectants were then selected by culturing cells in CM containing 200 U/ml hygromycin B (Calbiochem, San Diego CA). FACS was used to enrich the fraction of GFP-expressing cells from 15% to 60% (data not shown).
In vitro CTL cultures and cytotoxicity assays

Eleven to 14 days after immunization, animals were sacrificed, and a single-cell suspension of splenocytes was prepared. Splenocytes from DNA-primed animals were stimulated in vitro with each of the peptide epitopes represented in the minigene. Splenocytes (2.5–3.0 \( \times 10^7 \) /flask) were cultured in upright 25 cm\(^2\) flasks in CM containing 10 mg/ml peptide and 10 \( \times \) 10^7 irradiated spleen cells that had been activated for 3 days with LPS (25 \( \mu \)g/ml) and dextran sulfate (7 \( \mu \)g/ml). Triplicate cultures were stimulated with each epitope. Five days later, cultures were fed by replacing 7 ml of spent medium with fresh CM. After 10 days of in vitro culture, 2–4 \( \times \) 10^6 CTLs from each flask were harvested, washed, and restimulated in 6-well plates with 10^7 LPS/dextran sulfate-activated splenocytes that had been treated with 100 \( \mu \)g/ml peptide for 60–75 min at 37°C, then irradiated with 3500 rad. Eighteen hours later, Con A-activated splenocyte supernatant (10–15% final concentration, v/v) was added to cultures that were then

FIGURE 1. A. Nucleotide and amino acid sequence of epitopes encoded in pMin.1 DNA construct. The nucleotide sequences that flank the pMin.1 open reading frame are restriction sites for Nhel (A) and KpnI (C), and the Kozak consensus sequence (B). B. Expression vector for DNA minigene immunogenicity studies. pMin.1 and related minigene DNA were subcloned into the pcDNA3.1 expression vector.

In vitro CTL cultures and cytotoxicity assays

Eleven to 14 days after immunization, animals were sacrificed, and a single-cell suspension of splenocytes was prepared. Splenocytes from DNA-primed animals were stimulated in vitro with each of the peptide epitopes represented in the minigene. Splenocytes (2.5–3.0 \( \times 10^7 \) /flask) were cultured in upright 25 cm\(^2\) flasks in CM containing 10 \( \mu \)g/ml peptide and 10^7 irradiated spleen cells that had been activated for 3 days with LPS (25 \( \mu \)g/ml) and dextran sulfate (7 \( \mu \)g/ml). Triplicate cultures were stimulated with each epitope. Five days later, cultures were fed by replacing 7 ml of spent medium with fresh CM. After 10 days of in vitro culture, 2–4 \( \times \) 10^6 CTLs from each flask were harvested, washed, and restimulated in 6-well plates with 10^7 LPS/dextran sulfate-activated splenocytes that had been treated with 100 \( \mu \)g/ml peptide for 60–75 min at 37°C, then irradiated with 3500 rad. Eighteen hours later, Con A-activated splenocyte supernatant (10–15% final concentration, v/v) was added to cultures that were then
fed or expanded on the third day with CM-containing Con A supernatant. Five days after restimulation, CTL activity of each culture was measured by incubating varying numbers of CTLs with 10^4 35Cr-labeled target cells in the presence or absence of peptide. To decrease nonspecific cytotoxicity from NK cells, YAC-1 cells (American Type Culture Collection, Manassas, VA) were also added at a YAC-1:35Cr-labeled target cell ratio of 20:1.

To more readily compare responses, the standard E:T ratio versus percent cytotoxicity data curves were converted into lytic units (LU) per 10^6 effector cells with 1 LU defined as the lytic activity required to achieve 30% lysis of target cells. Specific CTL activity (ΔLU) was calculated by subtracting the LU value obtained in the absence of peptide from the LU value obtained with peptide. A given culture was scored positive for CTL induction if all of the following criteria were met: 1) ΔLU > 2; 2) LU (+ peptide) > LU (− peptide) > 3; and 3) a >10% difference in the percent cytotoxicity of target cells tested with and without peptide at the two highest E:T ratios (maximum E:T ratios were routinely between 25-50:1). CTL lines were generated from pMin.1-primed splenocytes through repeated weekly stimulations of CTLs with peptide-treated LPS/dextran sulfate-activated splenocytes using the 6-well culture conditions described above with the exception that CTLs were expanded in cytokine-containing CM as necessary during the 7-day stimulation period.

Cytokine assay
To measure IFN-γ production in response to minigene-transfected target cells, 4 × 10^5 CTLs were cultured with an equivalent number of minigene-transfected Jurkat-A2.1/Kb cells in 96-well flat-bottom plates. After overnight incubation at 37°C, culture supernatant from each well was collected and assayed for IFN-γ concentration using a sandwich ELISA. Immulon II microtiter wells (Dynatech, Boston, MA) were coated overnight at 4°C with 0.2 μg of anti-mouse IFN-γ capture Ab, R4-6A2 (PharMingen, San Diego, CA). After washing wells with PBS/0.1% Tween 20 and blocking with 1% BSA, Ab-coated wells were incubated with culture supernatant samples for 2 h at room temperature. A secondary anti-IFN-γ Ab, XMG1.2 (PharMingen), was added to wells and allowed to incubate for 2 h at room temperature. Wells were then developed by incubations with Avidin-DH and finally with biotinylated horseradish peroxidase H (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and TMB Laboratories, peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After washing wells with PBS/0.1% Tween 20 and blocking with 1% BSA, Ab-coated wells were incubated with culture supernatant samples for 2 h at room temperature. A secondary anti-IFN-γ Ab, XMG1.2 (PharMingen), was added to wells and allowed to incubate for 2 h at room temperature. Wells were then developed by incubations with Avidin-DH and finally with biotinylated horseradish peroxidase H ( Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and TMB Laboratories, peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The amount of cytokine present in each sample was calculated using a titrator standard.

Results
Selection of epitopes and minigene construct design
Nine CTL epitopes were chosen on the basis of their recognition by CTLs during HBV and HIV infection in humans, their sequence conservancy among viral subtypes, and their class I MHC binding affinity (Table I). Of these nine epitopes, six are restricted by HLA-A2.1 and three by HLA-A11. One epitope, HBV Pol 551, was studied in two alternative forms: either the wild-type sequence (HBV Pol 551-A) or an analogue (HBV Pol 551-V) engineered for higher binding affinity.

As indicated in Table I, several independent laboratories have reported that these epitopes are part of the dominant CTL response during HBV or HIV infection. All of the epitopes considered showed >75% conservancy in primary amino acid sequence among the different HBV subtypes and HIV clades. The MHC binding affinity of the peptides was also considered in selection of the epitopes. We wished to address the feasibility of immunizing with epitopes possessing a wide range of affinities and, as shown in Table I, the six HBV and three HIV HLA-restricted epitopes we selected covered a spectrum of MHC binding affinities spanning over two orders of magnitude, with IC50 concentrations ranging from 3 to 200 nM.

The immunogenicity of the six A2.1- and three A11-restricted CTL epitopes was verified by coimmunizing transgenic mice with each CTL epitope together with a Th cell epitope, both prepared in an IFA formulation. All of the epitopes induced significant CTL responses in the 5–73 ΔLU range (Table I). As mentioned above, to improve the MHC binding and immunogenicity of HBV Pol 551, the C-terminal A residue of this epitope was substituted with V, resulting in a 40-fold increase in binding affinity to HLA-A2.1 (Table I). While the parental sequence was weakly or nonimmunogenic in HLA transgenic mice, the HBV Pol 551-V analogue induced significant levels of CTL activity when administered in vivo. The parental sequence was weakly or nonimmunogenic in HLA transgenic mice, the HBV Pol 551-V analogue induced significant levels of CTL activity when administered in IFA (Table I). On the basis of these results, the V analogue of the HBV Pol 551 epitope was selected for the minigene construct. In all experiments, vivo-primed CTLs were stimulated in vitro against the native HBV Pol 551-A epitope and tested for cytolytic activity against target cells in the presence of the same peptide, irrespective of whether the V-containing analogue or native epitope was used for immunization.

Finally, because previous studies indicated that the induction of Th cell activity significantly improved the magnitude and duration of CTL responses (23, 37), the universal Th cell epitope PADRE was also incorporated into the minigene. PADRE has been shown previously to have high MHC binding affinity to a wide range of mouse and human MHC class II haplotypes (23). In particular, we have previously shown that PADRE is highly immunogenic in C57BL/6 mice that are used in the current study (23).

Table I. CTL epitopes in DNA minigene

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence</th>
<th>MHC Restriction</th>
<th>MHC Binding Affinity (IC50, nM)</th>
<th>Immunogenicity In Vivo (IFA)^ab</th>
<th>No. CTL-positive cultures^c</th>
<th>CTL response geo. mean ΔLU (^x± SD)d</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV Core 18</td>
<td>FLPSDFFPSV</td>
<td>A2.1</td>
<td>3</td>
<td>6/6</td>
<td>73.0 (1.1)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>HBV Env 335</td>
<td>WLSLVIFFV</td>
<td>A2.1</td>
<td>5</td>
<td>4/6</td>
<td>5.3 (1.6)</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>HBV Pol 455</td>
<td>GLSRVARL</td>
<td>A2.1</td>
<td>76</td>
<td>6/6</td>
<td>13.6 (1.4)</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>HIV Env 120</td>
<td>KITPLCVTL</td>
<td>A2.1</td>
<td>102</td>
<td>2/5</td>
<td>6.4 (1.3)</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>HIV Pol 476</td>
<td>ILKPEVPGV</td>
<td>A2.1</td>
<td>192</td>
<td>2/5</td>
<td>15.2 (2.9)</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>HBV Pol 551-A</td>
<td>YMDVVLGA</td>
<td>A2.1</td>
<td>200</td>
<td>0/6</td>
<td>–</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>HBV Pol 551-V</td>
<td>YMDVVLGV</td>
<td>A2.1</td>
<td>5</td>
<td>6/6</td>
<td>8.2 (2.3)</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>HIV Env 49</td>
<td>TVYGYVPVVK</td>
<td>A11</td>
<td>28/33</td>
<td>13.4 (3.1)</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV Core 141</td>
<td>STLPEETVVR</td>
<td>A11</td>
<td>4</td>
<td>6/6</td>
<td>12.1 (2.6)</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>HBV Pol 149</td>
<td>HTLWKGILYK</td>
<td>A11</td>
<td>14</td>
<td>6/6</td>
<td>13.1 (1.2)</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

^a Peptide tested in HLA-A2.1/Kb-H-2^d transgenic mice by coimmunizing with a Th cell peptide in IFA.
^b Number of CTL-positive cultures/$th$ tested. See Materials and Methods for criteria of positive cultures.
^c Geometric mean response of positive cultures as shown.
^d A. Sette, unpublished observation.
pMin.1, the prototype DNA minigene encoding nine CTL epitopes and PADRE, shown in Fig. 1A, was synthesized, subcloned into the pcDNA3.1 vector (Fig. 1B), and used for immunization studies. The mouse Igκ signal sequence was also included at the 5′ end of the construct to facilitate processing of the CTL epitopes in the ER as reported by others (19).

Immunogenicity of pMin.1 in transgenic mice

To assess the capacity of the pMin.1 minigene construct to induce CTLs in vivo, HLA-A2.1/Kb-H-2bw mice were immunized with pMin.1 DNA inserted in the pcDNA3.1 expression vector. Splenocytes from primed animals were cultured in triplicate flasks and stimulated twice in vitro with each peptide epitope. CTL induction against the HBV Pol 551-V analogue epitope encoded in pMin.1 was measured by stimulating cultures in vitro with the native HBV Pol 551-A epitope. Cytotoxicity of each flask was assayed in a 51Cr release assay against Jurkat-A2.1/Kb target cells in the presence (filled symbols, solid lines) or absence (open symbols, dotted lines) of peptide. Each symbol represents the response of a single flask culture.

FIGURE 2. Representative CTL responses against epitopes encoded in pMin.1 DNA. HLA-A2.1/Kb-H-2bw mice were immunized with pMin.1 DNA inserted in the pcDNA3.1 expression vector. Splenocytes from primed animals were cultured in triplicate flasks and stimulated twice in vitro with each peptide epitope. CTL induction against the HBV Pol 551-V analogue epitope encoded in pMin.1 was measured by stimulating cultures in vitro with the native HBV Pol 551-A epitope. Cytotoxicity of each flask was assayed in a 51Cr release assay against Jurkat-A2.1/Kb target cells in the presence (filled symbols, solid lines) or absence (open symbols, dotted lines) of peptide. Each symbol represents the response of a single flask culture.

pMin.1, the prototype DNA minigene encoding nine CTL epitopes and PADRE, shown in Fig. 1A, was synthesized, subcloned into the pcDNA3.1 vector (Fig. 1B), and used for immunization studies. The mouse Igκ signal sequence was also included at the 5′ end of the construct to facilitate processing of the CTL epitopes in the ER as reported by others (19).

Immunogenicity of pMin.1 in transgenic mice

To assess the capacity of the pMin.1 minigene construct to induce CTLs in vivo, HLA-A2.1/Kb-H-2bw transgenic mice were immunized i.m. with 100 μg of naked DNA. As a means of comparing the level of CTLs induced by DNA immunization, a control group of animals was also immunized with Theradigm-HBV, a palmitylated lipopeptide consisting of the HBV Core 18 CTL epitope linked to the tetanus toxin 830–843 Th cell epitope (24).

Splenocytes from immunized animals were stimulated twice in vitro with each of the peptide epitopes encoded in the minigene, then assayed for peptide-specific cytotoxic activity in a 51Cr release assay. A representative panel of CTL responses of pMin.1-primed splenocytes, shown in Fig. 2, indicates that significant levels of CTL induction were generated by minigene immunization. The cytolytic response in a majority of the cultures stimulated with the different epitopes exceeded 50% at an E:T ratio of 1:1. The strong CTL responses observed in pMin.1-primed animals was not due to primary CTL induction resulting from repeated in vitro stimulation with peptide inasmuch as splenocytes from animals immunized with an irrelevant minigene plasmid, pTol.1, did not show significant CTL induction above background following two in vitro stimulations with the HLA-A2.1-restricted epitopes encoded in pMin.1 (Fig. 3).

To more conveniently compare levels of CTL induction among the different epitopes, the percent cytotoxicity values for each splenocyte culture was converted to ΔLU, and the mean ΔLU of CTL activity in positive cultures for each epitope was determined.
(see Material and Methods for positive criteria). The average results of four independent experiments compiled in this manner are shown in Table II. CTL responses ranging between 50 to 700 ΔLU were routinely observed against the six A2.1-restricted epitopes. More significantly, the responses of several hundred ΔLU observed for five of the six epitopes approached or exceeded that of the Theradigm-HBV lipopeptide, a vaccine formulation known for its CTL-inducing potency in humans (24, 37). The HBV Env 335 epitope was the only epitope showing a lower mean ΔLU response than Theradigm-HBV (Table II, 44 vs 349 ΔLU).

Processing of minigenic epitopes by transfected cells

The decreased CTL response observed against HBV Env 335 was somewhat unexpected because this epitope had good A2.1 binding affinity (IC50 = 5 nM) and it was immunogenic in vivo when administered in IFA. We hypothesized that the lower response may be due, at least in part, to the inefficient processing of this epitope from the minigen polypeptide by APCs following in vivo DNA immunization. To address this possibility, Jurkat-A2.1/Kb tumor cells were transfected with pMin.1 DNA and the in vitro presentation of the HBV Env 335 epitope by transfected cells was compared with the other more immunogenic A2.1-restricted epitopes. Epitope presentation was also studied using tumor cells transfected with a second DNA construct, pMin.0-GFP, that encoded a similar multipeptide minigene fused with GFP. The latter APCs were enriched for minigene-expressing cells by FACS.

Epitope presentation by the transfected Jurkat cells was analyzed in vitro using specific CTL lines, with cytotoxicity or IFN-γ production serving as a read-out. It was found that the levels of CTL response in vitro correlated directly with the in vivo immunogenicity of the epitopes. Epitopes that were highly immunogenic in vivo, such as HBV Core 18, HIV Pol 476, and HBV Pol 457, were efficiently presented to CTL lines by pMin.1- or pMin.0-GFP-transfected cells, as measured by IFN-γ production (Fig. 4A); >100 pg/ml for each epitope) or cytotoxic activity (Fig. 4C, >30% specific cytotoxicity). In contrast to these high levels of in vitro activity, the stimulation of the HBV Env 335-specific CTL line against both populations of transfected cells resulted in <12 pg/ml IFN-γ (Fig. 4A) and 3% specific cytotoxicity (Fig. 4C). Although the HBV Env 335-specific CTL line did not recognize the naturally processed epitope efficiently, this line did show an equivalent response to peptide-loaded target cells, as compared with CTL lines specific for the other epitopes (Fig. 4, B and D). Collectively, these results suggest that inefficient processing and/or presentation of the HBV Env 335 epitope may contribute to its diminished immunogenicity in vivo.

Longevity of pMin.1 CTL responses

The longevity of the CTL responses induced by pMin.1 against five representative epitopes was examined next. Strong memory CTL responses could still be detected 18 wk after a single i.m. immunization. As shown in Table III, robust memory CTL responses exceeding 200 ΔLU were seen against Pol 551, Pol 476, and Core 18, while a lower but still significant response was observed against Env 335 (64 ΔLU in three of the three positive

<table>
<thead>
<tr>
<th>Epitope</th>
<th>No. positive cultures/total</th>
<th>Geo. mean ΔLU (×/± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV Core 18</td>
<td>9/9</td>
<td>455.4 (2.2)</td>
</tr>
<tr>
<td>HIV Env 120</td>
<td>12/12</td>
<td>211.9 (3.7)</td>
</tr>
<tr>
<td>HBV Pol 551-V</td>
<td>9/9</td>
<td>126.1 (2.8)</td>
</tr>
<tr>
<td>HBV Pol 455</td>
<td>12/12</td>
<td>738.6 (1.3)</td>
</tr>
<tr>
<td>HIV Pol 476</td>
<td>11/11</td>
<td>716.7 (1.5)</td>
</tr>
<tr>
<td>HBV Env 335</td>
<td>12/12</td>
<td>43.7 (1.8)</td>
</tr>
<tr>
<td>HBV Core 18</td>
<td>10/10</td>
<td>349.3 (1.8)</td>
</tr>
</tbody>
</table>

* Mice were immunized with pMin.1 DNA or Theradigm-HBV lipopeptide, and CTL activity in splenocyte cultures was determined after in vitro stimulation with individual peptide epitopes. Results from four independent experiments are shown.

** See Materials and Methods for criteria of a CTL-positive culture.

*** Geometric mean CTL response of positive cultures shown.

**** Response of mice immunized with Theradigm-HBV lipopeptide containing the HBV Core 18 epitope.
cultures). For the Core 18 epitope, the magnitude of memory CTL response induced by minigene DNA was comparable to that induced by lipopeptide. The CTL response appeared to have waned against HBV Pol 455, because only one of three cultures showed positive CTL induction. The consistency of this response increased to 100% positive cultures following a booster immunization with pMin.1. Boosting also led to a fourfold increase in the Pol 551 response, but it did not increase responses to the other four epitopes, three of which already displayed strong memory CTL responses following a single immunization.

### Effect of the Th cell epitope PADRE on minigene immunogenicity

Having obtained a broad and balanced CTL response in transgenic mice immunized with a minigene DNA encoding multiple HLA-A2.1-restricted epitopes, we next examined possible variables that could influence the immunogenicity of the prototype construct. We reasoned that this analysis could provide the foundation for rational and rapid optimization of future constructs. To examine the contribution of T cell help in minigene immunogenicity, a DNA construct based on the pMin.1 prototype was synthesized in which the PADRE epitope was deleted (Fig. 5A).

The results of the in vivo immunogenicity analysis indicated that the deletion of PADRE resulted in significant decreases in the frequency of specific CTL precursors against four of the minigene epitopes (HBV Core 18, HIV Env 120, HBV Pol 455, and HBV Env 335) as indicated by the 17–50% CTL-positive cultures observed against these epitopes compared with the 90–100% frequency in animals immunized with the prototype pMin.1 construct (Fig. 6A). Moreover, for two of the epitopes, HBV Core 18 and HIV Env 120, the magnitude of the CTL response in positive cultures induced by pMin.1-No PADRE was 20- to 30-fold less than that the responses induced by the pMin.1.

### Effect of modulating MHC binding affinity on epitope immunogenicity

To address the effect of decreasing MHC binding on epitope immunogenicity, we synthesized a construct in which the V anchor residue in HBV Pol 551 was replaced with A, the native residue (Fig. 5B). Unlike deletion of the Th cell epitope, decreasing the MHC binding capacity of the HBV Pol 551 epitope by 40-fold through modification of the anchor residue did not appear to affect epitope immunogenicity. The CTL response against the HBV Pol 551 epitope, as well as to the other epitopes, measured either by LU or

---

### Table III. Memory CTL responses in pMin.1-immunized mice

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Single Immunization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>With Booster Immunization&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive cultures&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CTL response geo. mean DLU (×/± SD)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>HBV Core 18</td>
<td>3/3</td>
<td>655.1 (2.9)</td>
</tr>
<tr>
<td>HBV Pol 551-V</td>
<td>3/3</td>
<td>211.7 (2.8)</td>
</tr>
<tr>
<td>HBV Pol 455</td>
<td>1/3</td>
<td>159.0 –</td>
</tr>
<tr>
<td>HIV Pol 476</td>
<td>3/3</td>
<td>865.4 (1.1)</td>
</tr>
<tr>
<td>HBV Env 335</td>
<td>3/3</td>
<td>64.1 (1.5)</td>
</tr>
<tr>
<td>HBV Core 18 (theradigm-HBV)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3/3</td>
<td>674.7 (1.6)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice were immunized with 100 μg pMin.1 DNA, and 18 wk later splenocytes were stimulated twice in vitro with each peptide epitope before CTL activity was measured.

<sup>b</sup> Mice were immunized with 100 μg pMin.1 DNA and boosted 8 wk later with an identical amount of DNA. Then, 18 wk after primary immunization, splenocytes were stimulated twice in vitro with each peptide epitope.

<sup>c</sup> Number of CTL-positive cultures/total tested. See Materials and Methods for criteria of a positive culture.

<sup>d</sup> Geometric mean response of CTL-positive cultures are shown.

<sup>e</sup> Response of mice immunized with Theradigm-HBV lipopeptide containing the HBV Core 18 epitope after 18 wk postimmunization.

---

**FIGURE 5.** Modified minigene constructs that address variables critical for in vivo immunogenicity. The following modifications were incorporated into the prototype pMin.1 construct: A, deletion of PADRE Th cell epitope; B, incorporation of the native HBV Pol 551 epitope that contains an A in position 9; C, deletion of the Ig κ signal sequence; and D, switching position of the HBV Env 335 and HBV Pol 476 epitopes. All of the indicated DNA minigenes were subcloned into the pcDNA3.1 expression vector and used for immunization.
frequency of CTL-positive cultures, was very similar between the constructs containing the native A or improved V residue, at least in the presence of the PADRE Th epitope and at a 100-μg priming dose (Fig. 6B). This finding reinforces the notion that minimal epitope minigenes can efficiently deliver epitopes of vastly different MHC binding affinities. Furthermore, this finding is particularly relevant to enhancing epitope immunogenicity via different delivery methods, since the wild-type HBV Pol 551 epitope was essentially nonimmunogenic when delivered as free peptide in an IFA emulsion (Table I).

**Effect of the signal sequence on minigene construct immunogenicity**

The signal sequence was deleted from the pMin.1 construct, thereby abolishing ER-targeting of the minigene polypeptide (19) (Fig. 5C). When the immunogenicity of the pMin.1-No Sig construct was examined, we found an overall decrease in response against four CTL epitopes. Two of these epitopes, HIV Env 120 and HBV Env 335, showed a decrease in frequency of CTL-positive cultures compared with pMin.1, while HBV Pol 455 and HIV Pol 476 showed a 16-fold (from 424 to 27 ΔLU) and 3-fold decrease (709 to 236 ΔLU) in magnitude of the mean CTL response, respectively (Fig. 6C). These findings suggest that allowing ER-processing of some of the epitopes encoded in the pMin.1 prototype construct may improve immunogenicity, as compared with constructs that allow only cytoplasmic processing of the same panel of epitopes. Alternatively, inefficient processing and presentation of the PADRE epitope in the signal-deleted construct may have affected CTL induction.

**Effect of epitope rearrangement and creation of new junctional epitopes**

In the final construct tested, we analyzed whether the immunogenicity of the HBV Env 335 epitope may be influenced by its position at the 3′ terminus of the minigene construct (Fig. 5D). The position of the Env 335 epitope in the DNA construct was switched with a more immunogenic epitope, HBV Pol 455, located in the center of the minigene. It should be noted that, as discussed below, this modification also created two potentially new epitopes containing junctional sequences.

As shown in Fig. 6D, the transposition of the two epitopes appeared to affect the immunogenicity of not only the transposed epitopes but also, more globally, the immunogenicity of other epitopes. Switching of the two epitopes resulted in obliteration of CTL induction against HBV Env 335 (no positive cultures detected of six). The CTL response induced by the terminal HBV Pol 455 epitope was also decreased from a mean of 424 ALU to 78; however, the 78 ALU response in all six positive cultures was still considered to be a significant level of CTL induction. In addition to the switched epitopes, CTL induction against other epitopes in the pMin.1-Switch construct was also markedly reduced compared with the prototype construct. For example, a CTL response was not observed against the HIV Env 120 epitope, and responses were significantly diminished against HBV Core 18 (four of six positive cultures, decrease in mean ALU from 306 to 52) and against HBV Pol 476 (decrease from 709 ALU to 20) (Fig. 6D). It should be noted that switching of the epitopes created new junctional epitopes between HBV Env 335-HIV Pol 476 (HLVPVFIL, H-2Kb-restricted) and HBV Env 335-HBV Pol 551 (VL GVWLSLLV, HLA-A2.1-restricted). Although we have not examined whether these junctional epitopes are indeed immunogenic, they may account for the low immunogenicity of the Env 335 and Pol 476 epitopes. These findings suggest that avoiding junctional epitopes may be important in designing multiepitope minigenes, as is the ability to confirm their immunogenicity in vivo in a biological assay system such as HLA transgenic mice.

**Relative potency of multiepitope minigene DNA vs whole gene DNA for epitope-specific CTL induction**

One obvious advantage of a multiepitope minigene DNA vaccine is its flexibility inasmuch as only a single vaccine construct would be required to induce CTL immunity against epitopes from different protein Ags. In contrast, the generation of a similarly broad CTL response through vaccination with DNA encoding whole proteins will require a mixture of genes. Although both forms of DNA have been shown to induce CTLs, there is a paucity of information regarding the relative potency of epitope-specific CTL induction by immunization with a multiepitope minigene vs whole protein gene. We addressed this question by comparing the CTL response against two dominant HLA-A2.1-restricted HBV polymerase epitopes following immunization with varying doses of pMin.1 or with DNA encoding the entire polymerase protein. The expression vector used for immunization of both forms of DNA was identical, namely the pcDNA 3.1 vector. The results, shown in Table IV,
indicate that there was a significant difference in the immunogenicity of two DNA constructs. Strong CTL induction was observed following minigene immunization, with significant responses still observed with as little as 250 ng of DNA. In contrast, polymerase DNA immunization, even at a 100-μg dose, induced very little CTLs against the HBV Pol 551 epitope. In the case of the HBV Pol 455 epitope, 100 μg of DNA encoding the whole polymerase protein induced a response that was 10-fold lower (68.6 vs 741.2 ΔLU) than pMin.1 DNA. Based on the amount of DNA necessary to induce equivalent responses, it appears that the immunogenicity of the pMin.1 minigene construct exceeded the whole gene by more than 20-fold (Table IV).

Induction of CTLs against HLA-A11-restricted epitopes encoded in pMin.1

To further examine the flexibility of the minigene vaccine approach for inducing a broad CTL response against multiple epitopes restricted by different HLA alleles, we immunized HLA-A11/Kb transgenic mice to determine whether the three A11 epitopes in the pMin.1 construct were immunogenic for CTLs, as was the case for the A2.1-restricted epitopes in the same construct. As summarized in Table V, significant CTL induction directed against all three of the HLA-A11-restricted epitopes was observed in a majority of cultures, and the level of CTL immunity, in the range of 40 to 260 ΔLU, exceeded that of the respective peptides delivered in IFA (Table I). Thus, nine CTL epitopes of varying HLA restrictions incorporated into a prototype minigene construct all demonstrated significant CTL induction in vivo, confirming that minigene DNA plasmids can serve as means of delivering multiple epitopes, of varying HLA restrictions and MHC binding affinities, to the immune system in an immunogenic fashion and that appropriate transgenic mouse strains can be used to measure DNA construct immunogenicity in vivo.

Discussion

This study represents the first description of the use of HLA transgenic mice (21, 22, 27) for quantitating the in vivo immunogenicity of DNA vaccines encoding HLA-restricted CTL epitopes. Herein, we investigated the possibility of inducing CTL responses directed against multiple dominant HLA-A2.1- and A11-restricted epitopes encoded in a DNA plasmid. In addition to validating the transgenic animal system as a convenient and rapid way to assay and optimize DNA minigene constructs destined for human use, we have used the HLA transgenic mouse model system to address fundamental variables that may affect the in vivo potency of minigene vaccines such as epitope affinity to MHC, T cell help, signal sequence, and epitope position.

Multiepitope plasmids containing murine MHC class I-, class II-, and Ab-binding epitopes, delivered by immunizing with naked DNA or by infecting with recombinant viruses, have been shown to be immunogenic in mice and to confer protection against viral infection and cancer (7–12). However, the MHC binding affinity of epitopes used in these studies was not quantitated. The results presented here are particularly significant because a strong and balanced CTL response could be achieved in HLA transgenic mice against all epitopes encoded in our minigene, even though their MHC binding affinities ranged over approximately two orders of magnitude, and, in the case of one epitope, HBV Pol 551, immunogenicity was apparently unaffected by a 40-fold variation in binding affinity.

This finding demonstrates the flexibility of the multiepitope approach, even in the case of vaccines that include epitopes of vastly different MHC affinity. Furthermore, they suggest that even in response to the high immunogen doses used in this study, competition for peptide-receptive class I MHC molecules might not be a factor of major importance in determining immunodominance in vivo. It remains to be determined whether this finding is generalizable to other constructs encoding epitopes with varying MHC binding affinities, including perhaps subdominant epitopes, and what the lower limits of MHC binding affinity for immunogenicity in a minigene might be.

When other variables besides MHC binding affinity were examined, it appeared that immunogenicity of at least some of the epitopes in the minigene was dependent on T cell help. Deletion of

<table>
<thead>
<tr>
<th>Epitope</th>
<th>No. positive cultures/total</th>
<th>Geo. mean ΔLU (×/± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV Core 141</td>
<td>5/9</td>
<td>128.1 (1.6)</td>
</tr>
<tr>
<td>HBV Pol 149</td>
<td>6/9</td>
<td>267.1 (2.2)</td>
</tr>
<tr>
<td>HIV Env 49</td>
<td>9/9</td>
<td>40.1 (2.9)</td>
</tr>
</tbody>
</table>

*a Mice were immunized with pMin.1 DNA, and CTL activity in splenocyte cultures was determined after in vitro stimulation with individual A11-restricted peptide epitopes. The geometric mean CTL responses from three independent experiments are shown.

*b Criteria for CTL-positive culture is described in Materials and Methods.

c Geometric mean CTL response of positive cultures shown.
the PADRE Th cell epitope from the prototype construct inhibited CTL responses to four of the six epitopes tested, as demonstrated by a decrease in frequency and magnitude of the CTL responses. Measurement of T cell lymphoproliferation in primed splenocytes revealed that PADRE-specific Th cells were induced in animals immunized with the prototype pMin.1 construct but were absent in animals immunized with the PADRE-deleted construct (data not shown).

These results are in apparent contrast with the findings reported by Thomson et al. (11), who were able to induce CTLs in anti-CD4-treated animals after immunization with a multiepitope DNA minigene. However, it should be noted that the responses in Th cell-depleted mice to some of the epitopes in the minigene construct of Thomson et al. (11) appeared to be inhibited in magnitude as compared with animals treated with a control Ab. Previous results in both murine and human systems indicated that, while CTL induction is not absolutely dependent on T cell help, immunization with vaccine constructs containing a Th and CTL epitopes did result in markedly augmented CTL activity (16, 24), and recent data illustrate a critical role for CD40 up-regulation on dendritic cells in mediating help for CTL responses (38–40).

It is unclear why a more global inhibition of CTL induction encompassing all of the epitopes was not observed with the PADRE-deleted minigene construct. The possibility that some level of Th cell activity, in the absence of a Th cell epitope might be provided by the adjuvant effect of immunostimulatory sequences in the DNA plasmid should also be considered (41). Because the potency of CpG sequences in humans is still unproven, development of vaccines with optimized Th cell function appears to be prudent at this time.

Another variable in construct design that was found to affect immunogenicity was the presence of an ER-translocating signal sequence. Such signal sequences target processing of proteins to the ER, where they are degraded into peptides and loaded onto class I MHC molecules (19). Herein, we compared the immunogenicity of our prototype construct with and without an Ig κ-chain signal sequence. The results indicated that, in the absence of a signal sequence, CTL induction to four of six epitopes was decreased compared with an otherwise identical construct containing a signal sequence. Thus, our studies confirm the results by Anton et al. (42), which show that ER-targeted minigenes encoding short peptide fragments are efficiently processed in the ER, and extend their findings to processing of larger multiepitope minigene products.

As previously mentioned, we observed vigorous CTL induction against all epitopes encoded in our prototype minigene construct. However, it should be noted that the response against one epitope, HBV Env 335, was 3- to 17-fold lower than the others. This effect was not due to suboptimal binding affinity (Table I) or to a deletion in the T cell repertoire. Rather, the pattern of in vitro CTL recognition observed suggested that the Env 335 epitope was processed relatively inefficiently as compared with other epitopes that showed higher in vivo immunogenicity.

Flanking regions and the specific molecular context of a given epitope have been reported to influence immunogenicity in certain cases (13, 14) but not others (11, 15). We synthesized a modified construct where the position of the weaker Env 335 epitope was switched with that of a more immunogenic epitope, HBV Pol 455, located in the middle of the minigene. When this construct was tested in transgenic mice, Env 335 immunogenicity was obliterated and the responses against two other epitopes, HIV Env 120 and HIV Pol 476, were also inhibited. In contrast, the immunogenicity of the HBV Pol 455 epitope moved to the 3′ position of the minigene, was only moderately affected. Because switching of the two epitopes may have created new junctional CTL epitopes, the processing of the junctional epitopes may have resulted in the destruction of the HBV Env 335 and HIV Pol 476 epitopes and the diminished CTL responses against them. This hypothesis is currently being examined experimentally.

In the current study, we also examined the relative potency of minigene DNA compared with immunization against DNA encoding a whole viral Ag. In a priming dose titration study, it was evident that multiepitope minigene immunization resulted in stronger and more consistent induction of CTLs specific for epitopes in the HBV polymerase protein than immunization with whole protein-encoding DNA. Based on the observed dose response, minigene priming was at least 20-fold more effective than whole gene DNA priming, with significant responses still being observed with as little as 250 ng of DNA. This difference in relative potency could not be accounted for by differences in the m.w. of the gene products as both are similar in this regard (data not shown). We have not measured the relative levels of gene product synthesized in either pMin.1- or pc3.1-Pol-transfected cells, so the possibility exists that the differences between their in vivo immunogenicity may be due to differences in the level or quality of gene product expression. Although we have examined only a single viral protein, this apparent difference in immunogenicity of multiepitope vs whole protein DNA may indicate another important advantage of multiepitope minigene vaccines, in addition to their capacity to immunize with several epitopes from diverse Ags with a single construct.

With respect to multiepitope minigene DNA potency compared with other vaccine delivery systems, it should be noted that the magnitude of CTL induction observed for five of the HLA A2.1-restricted epitopes approximated that observed with Theradigm-HBV, a lipopeptide previously shown to induce strong CTL responses in humans (24, 37). Furthermore, minigene immunization also induced strong memory CTL responses against several of the epitopes that could be detected up to 18 wk after primary immunization.

Lastly, we would also like to point out that not only were CTL responses induced against six A2.1-restricted epitopes in A2.1/Kb- H-2 bxs transgenic mice immunized with the pMin.1 construct, but they were also induced against three A11-restricted epitopes in A11/K b transgenic mice. These responses suggest that minigene delivery of multiple CTL epitopes that confers broad population coverage may be possible in humans and that transgenic animals of appropriate haplotypes may be a useful tools in optimizing the in vivo immunogenicity of minigene DNA.

As mentioned above, this study represents the first description of the use of HLA transgenic mice to quantitate the in vivo immunogenicity of DNA vaccines. In vivo studies are required to address the variables crucial for vaccine development that are not easily evaluated by in vitro assays, such as route of administration, vaccine formulation, tissue biodistribution, and involvement of primary and secondary lymphoid organs. Because of its simplicity and flexibility, the use of HLA transgenic mice might represent an attractive alternative, at least for initial vaccine development studies, compared with more cumbersome and expensive studies in higher animal species, such as nonhuman primates. The in vitro presentation studies described above further supports the use of HLA transgenic mice for screening DNA constructs containing human epitopes inasmuch as a direct correlation between in vivo immunogenicity and in vitro presentation was observed. In light of the strong in vivo CTL responses observed against all of the HLA A2.1- and A11-restricted epitopes encoded in our prototype pMin.1 construct, it thus appears that multiepitope minigenes optimized by the use of HLA transgenic mice could be considered for
References


