Priming Biologically Active Antibody Responses Against an Isolated, Conformational Viral Epitope by DNA Vaccination

Petra Riedl, Shereen El K holy, Jörg Reimann and Reinhold Schirmbeck

*J Immunol* 2002; 169:1251-1260; doi: 10.4049/jimmunol.169.3.1251

http://www.jimmunol.org/content/169/3/1251

---

**References**

This article cites 49 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/169/3/1251.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
Priming Biologically Active Antibody Responses Against an Isolated, Conformational Viral Epitope by DNA Vaccination

Petra Riedl, Shereen El Kholy, Jörg Reimann, and Reinhold Schirmbeck

The immunodominant, conformational “a” determinant of hepatitis B surface Ag (HBsAg) elicits Ab responses. We selectively expressed the Ab-binding, glycosylated, native a determinant (residue 120–147) of HBsAg in a fusion protein containing C-terminally the HBsAg fragment SII (residue 80–180) fused to a SV40 T-Ag-derived hsp73-binding 77 aa (T77) or non-hsp-binding 60 aa (T60) N terminus. A DNA vaccine encoding non-hsp-binding secreted T60-SII fusion protein-stimulated murine Ab responses with a similar efficacy as a DNA vaccine encoding the secreted, native, small HBsAg. A DNA vaccine encoding hsp73-binding, intracellular T77-SII fusion protein-stimulated murine Ab responses less efficiently but comparable to a DNA vaccine encoding the intracellular, native, large HBsAg. HBsAg-specific Abs elicited by either the T60-SII-expressing or the T77-SII-expressing DNA vaccine suppressed HBsAg antigenemia in transgenic mice that produce HBsAg from a transgene in the liver; hence, a biologically active B cell response cross-reacting with the native, viral envelope epitope was primed by both DNA vaccine constructs. HBsAg-specific Ab and CTL responses were coprimed when an S20–50 fragment (containing the immunodominant, Ld-binding epitope S28–39) of HBsAg was fused C-terminally to the pCI/T77-SII sequence (pCI/T77-SII-Ld DNA vaccine). Chimeric, polyepitope DNA vaccines encoding conformational, Ab-binding epitopes and MHC class I-binding epitopes can thus efficiently deliver antigenic information to different compartments of the immune system in an immunogenic way. The Journal of Immunology, 2002, 169: 1251–1260.

Most viral epitopes that elicit protective Ab responses and should therefore be targeted by vaccines are conformational. Incorporation of intact conformational epitopes into protein- or DNA-based vaccines that stimulate protective Ab responses is difficult because expression of antigenic fragments containing conformational, Ab-binding epitopes are difficult to express by recombinant DNA. Purified recombinant proteins often lack the epitope because they are not subjected to the relevant posttranslational modifications in the expression system used or are partially denatured in the production process. It is therefore of interest to design systems that support selective expression of conformational, Ab-binding epitopes of vaccine-relevant Ags and their incorporation with other T or B cell-stimulating determinants into polyvalent vaccines.

Hepatitis B surface Ag (HBsAg) secreted by HBV-infected hepatocytes and present in the plasma of infected individuals is a complex macromolecule composed of proteins and lipids (1, 2). The 22-nm coreless HBsAg lipoprotein particles contain three natural variants of HBsAg designated large (LS), middle (MS), and small (S) surface proteins. The major HBsAg species is the small, secreted S protein present as nonglycosylated p24 and glycosylated gp27. The MS protein contains an additional N-terminal preS2 domain and is expressed as nonglycosylated p33 and glycosylated gp36. The intracellular LS protein with additional preS1 and preS2 domains N-terminal to the S region is expressed as nonglycosylated p39 and glycosylated gp42 (3). All three HBsAg species form particulate structures when expressed individually (2–4). The MS and S Ags are efficiently secreted from transfected cells, whereas the LS Ag is exclusively found intracellularly (3). All three natural HBsAg variants contain the conformational “a” determinant in the S120–147 region that is recognized by most human and murine anti-HBsAg Abs (2, 4–6). The a determinant of the S protein forms a loop between two transmembrane domains, is glycosylated, and is present in envelope proteins of almost all known HBV isolates. We investigated whether a HBsAg-derived sequence encoding the a determinant can be expressed in a DNA vaccine as either an intracellular, chimeric Ag (mimicking LS), or a secreted Ag (mimicking S), and can induce conformation-specific, biologically active Ab responses.

We used an expression system in which N-terminal, papovavirus (SV40)-derived sequences facilitate expression of protein fragments fused C-terminally to it (7). The N-terminal residues 1–60, 1–77, or 1–272 from SV40 T-Ag support expression of the a determinant-containing HBsAg fragment (residue 80–180) fused C-terminally to it. By varying the length of the N-terminal T-Ag-derived sequence, we changed the expression pattern of the chimeric protein with the a epitope of HBsAg resembling either the intracellular LS expression (stably associated with the hsp73 chaperone) or the secreted S expression (not associated with hsp). Expression was intracellular and hsp-associated when the N-terminal T-Ag sequence contained residue 1–77 with the intact, hsp-binding J domain (8, 9). Expression of a secreted product was obtained when the N-terminal T-Ag sequence contained T-Ag residue 1–60 with a disrupted, hsp-binding J domain.

Institute of Medical Microbiology and Immunology, University of Ulm, Ulm, Germany

Received for publication January 18, 2002. Accepted for publication June 3, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the German Federal Ministry for Science and Technology (01GE9907), the Deutsche Forschungsgemeinschaft (DFG Schi 505/2-1), and the University of Ulm (IZKF/A10) to R.S. and J.R.

2 P.R. and S.E.K. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Reinhold Schirmbeck, Institute for Medical Microbiology and Immunology, University of Ulm, Albert Einstein Allee 11, D-89081 Ulm, Germany. E-mail address: reinhold.schirmbeck@medizin.uni-ulm.de

4 Abbreviations used in this paper: HBsAg, hepatitis B surface Ag; HBV, hepatitis B virus; S, small HBsAg; MS, middle (preS2-S) HBsAg; LS, large (preS1-preS2-S) HBsAg; CHO, Chinese hamster ovary; Endo H, endoglycosidase H; ER, endoplasmic reticulum.
A critical question is whether the Ab response to the HBsAg a epitope elicted by the novel vaccine constructs is biological functional. HBV neutralization assays are not feasible in mice. We therefore tested whether the elicited Abs can suppress antigenemia (in mice expressing transgene-encoded HBsAg in the liver) after adoptive transfer of either serum or immune cells. This system allowed us to evaluate the potential for neutralization that the elicited Ab response can convey.

The efficient expression of the conformational B cell epitope, the testing of its immunogenicity in mice in vivo by DNA vaccination, and the evaluation of its potential neutralizing activity in transgenic mice indicated that the approach supports construction of polypeptidene DNA vaccines encoding chimeric Ags. We further used this system to express a more complex polypeptide vaccine containing B and T cell-stimulating epitopes. In this pCI/T77-SII-L'-L'1 vaccine, the MHC class I (L6'-binding epitope of HBsAg was fused C-terminally to the chimeric T77-SII Ag. This generated a vaccine that efficiently primed CTL and showed enhanced immunogenicity of the conformational B cell epitope. These data indicate that polypeptide DNA vaccines can efficiently coprime different compartments of the immune system and thus are a feasible option for the construction of complex, multivaccine vaccines.

**Materials and Methods**

**Mice**

BALB/cJ-Bom (H-2d) mice and C57BL/6J-Bom (H-2b) mice were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). C57BL/6J-TgN(Alb1HBV)44Bri transgenic (HBs-tg) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were used at 10–16 wk of age. Male and female mice were used at 12–16 wk of age.

**Cells**

The H-2d mastocytoma cell line P815 (TIB64) and the Chinese hamster ovary (CHO) cell line (CRL-1772) were obtained from the American Type Culture Collection (Manassas, VA). The chicken hepatoma cell line LHM was obtained from Dr. H.-J. Schlicht (University of Ulm, Institute for Virology, Ulm, Germany).

**Vector constructs**

Construction of the pCI vectors containing cT77, S, and LS has been described (10–12). pC/SII: The SII (SII1-n188) fragment was amplified from a HBsAg-encoding sequence by using a forward primer encoding a methionine start codon and a SalI site (AAAGCTGACATGATCCTTCCTTCATCCTGCTG) and a reverse primer encoding a stop signal and a NotI site (GGAAAAAGCCGCGCCTTCAAAATGGCACTAGTGAAACT). The amplified sequence cloned into the pCI vector (catalog no. E1731; Promega, Madison, WI) was used for DNA immunization. pC/T77-SII: The HBV sequence, encoding the HBV-S amino acid sequence, was fused in-frame to the N-terminal, hsp73-binding cT-Ag272 fragment. The CCCACTGGTATGAGAAATCCATACATATGAGA180 (SII) of HBsAg, was fused-in-frame to the hsp73-binding cT-Ag272 fragment.

For transient expression, LMH cells were transfected with recombinant pcDNA-based plasmids using the CaPO4 method. About 5 × 106 cells were seeded in 16-h after transfection, metabolically labeled for 12 h with 100 μCi [35S]methionine (catalog no. SJ1015; Amersham, Braunschweig, Germany), and extracted with 1 ml lysis buffer (100 mM NaCl, 1% aprotinin (Trasylol; Bayer, Leverkusen, Germany), leupeptin, 0.5% Nonidet P-40, and 100 mM Tris-HCl (pH 8.0) for 30 min at 4°C. Extracts were cleared by centrifugation (30 min, 14,000 rpm) and immunoprecipitated for T-Ag using 10 μl of mAb PAb108 (10 mg/ml stock) directed against the extreme N terminus of the T-Ag (15) and a gift from the Research Institute for Virology, Marburg, Germany). hsp was detected with the hsp73-specific Abs SPA-816 and SPA-818 or with the grp78- and grp94-specific Abs SPA-826 and SPA-851 (StressGen Biotechnologies, Victoria, Canada).

**DNA vaccination**

Two aliquots of plasmid DNA were injected into the two contralateral tibialis anterior muscles. For i.m. nucleic acid immunization, we injected 50 μl PBS containing 1 μg/ml plasmid DNA as described elsewhere (7, 11).

**Transient and stable expression of fusion proteins**

For transient expression, LHM cells were transfected with recombinant pCI-based plasmids using the CaPO4 method. About 5 × 106 cells were seeded in 16 h after transfection, metabolically labeled for 12 h with 100 μCi [35S]methionine (catalog no. SJ1015; Amersham, Braunschweig, Germany), and extracted with 1 ml lysis buffer (100 mM NaCl, 1% aprotinin (Trasylol; Bayer, Leverkusen, Germany), leupeptin, 0.5% Nonidet P-40, and 100 mM Tris-HCl (pH 8.0) for 30 min at 4°C. Extracts were cleared by centrifugation (30 min, 14,000 rpm) and immunoprecipitated for T-Ag using 10 μl of mAb PAb108 (10 mg/ml stock) directed against the extreme N terminus of the T-Ag (15) and a gift from the Research Institute for Virology, Marburg, Germany).

**ATP treatment of immunoprecipitated T-hsp73 complexes**

The T-hsp73 complexes were immunoprecipitated with the anti-T-Ag mAb PAb108 and protein A-Sepharose. Immune complexes were extensively washed as described above and treated for 15 min with 10 mM ATP or ADP (catalogue nos. A-2383 and A-2754, respectively; Sigma-Aldrich, St. Louis, MO). Samples were analyzed by SDS-PAGE and Western blot analysis. For transient expression, LMH cells were transfected with recombinant pcDNA-based plasmids using the CaPO4 method. About 5 × 106 cells were seeded in 16 h after transfection, metabolically labeled for 12 h with 100 μCi [35S]methionine (catalog no. SJ1015; Amersham, Braunschweig, Germany), and extracted with 1 ml lysis buffer (100 mM NaCl, 1% aprotinin (Trasylol; Bayer, Leverkusen, Germany), leupeptin, 0.5% Nonidet P-40, and 100 mM Tris-HCl (pH 8.0) for 30 min at 4°C. Extracts were cleared by centrifugation (30 min, 14,000 rpm) and immunoprecipitated for T-Ag using 10 μl of mAb PAb108 (10 mg/ml stock) directed against the extreme N terminus of the T-Ag (15) and a gift from the Research Institute for Virology, Marburg, Germany).

**Endoglycosidase (Endo H) treatment**

Endo H (catalog no. 1643053; Roche, Mannheim, Germany) digestion was performed on immunoprecipitated samples as described elsewhere (17). Protein A-Sepharose beads carrying ~0.5 μg mAb108-bound cT-SII Ags were resuspended in 60 mM sodium citrate (pH 5.5), 0.2% SDS, 30 μg BSA, 1% aprotinin, and leupeptin. After addition of 125 mM Endo H, the samples were incubated for 18 h at 37°C. After washing steps with PBS and 0.1% PBS, the proteins were recovered from protein A-Sepharose and processed for SDS-PAGE.

**Cytotoxic assays**

Single-cell suspensions were prepared from spleens of mice in α-MEM tissue culture medium supplemented with 10 mM HEPES buffer, 5 × 10−3 M 2-ME, antibiotics, and 10% v/v FCS (PAA Laboratories; Linz, Austria). A selected batch of Con A-stimulated rat spleen cell supernatant (2% v/v) was added to the culture medium. Three × 106 responder cells were cocultured with 1 × 106 irradiated, syngeneic HBsAg-expressing transfectants. Coculture was performed in 10 ml medium in upright 25-cm2 tissue culture
flasks in a humidified atmosphere of 5% CO2 at 37°C. After 5 days of culture, CTL were harvested, washed, and assayed for specific cytolytic reactivity. Serial dilutions of effector cells were cultured with 2 × 103 51Cr-labeled targets in 200-μl round-bottom wells. Specific cytolytic activity of cells was tested in 51Cr release assays against transfected, HBsAg-expressing P815/S targets or control P815 targets. After a 4-h incubation at 37°C, 50 μl of supernatant was collected for gamma radiation counting. The percentage of specific release was calculated as ((experimental release – spontaneous release)/(total release – spontaneous release)) × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always <15% of the total counts. Data shown are the mean of triplicate cultures. The SEM of triplicate cultures was always <20% of the mean.

**Determination of HBsAg-specific serum Ab levels and serum HBsAg**

Serum samples were repeatedly obtained from individual immunized or control mice by tail bleedings at different time points postinjection. Abs against HBsAg were detected in mouse sera using the commercial IMxAUSAB test (catalog no. 7A39-20; Abbott Laboratories, Wiesbaden, Germany) that detects exclusively the conformational α determinant. Abs against HBsAg were detected in mouse sera using the commercial AXSYM HBsAg (V2) kit (catalogue no. 7A40-22; Abbott Laboratories).

HBsAg-specific IgG1 and IgG2a serum Abs were determined by endpoint dilution ELISA. Briefly, microELISA plates (Nunc Maxisorp; Nunc, Wiesbaden, Germany) were coated with 150 ng rHBsAg/well in 50 μl 0.1 M sodium carbonate buffer (pH 9.5) at 4°C. Serial dilutions of the sera in loading buffer (PBS supplemented with 3% BSA, BSA, and 2% Tween 20) were added to the Ag-coated wells. Serum Abs were incubated for 2 h at 37°C followed by four washes with PBS supplemented with 0.05% Tween 20. Bound serum Abs were detected using HRP-conjugated rat anti-mouse IgG, IgG1, or IgG2a Abs (BD PharMingen, Hamburg, Germany) at a dilution of 1/2000 followed by incubation with o-phenylenediamine × 2 HCl (catalog no. 6172-24; Abbott Laboratories) in PBS (pH 6.0). The reaction was stopped by 1 M H2SO4 and the extinction was determined at 492 nm. End point titers were defined as the highest serum dilution that resulted in an absorbance value three times greater than that of negative control sera (derived from nonimmunized BALB/c mice).

**Results**

**Expression of secreted (S) and intracellular (LS) HBsAg variants**

We cloned HBsAg sequences encoding either the LS protein or the 226-residue small S protein into the pCI vector (Fig. 1A). We furthermore cloned HBsAg fragments encoding Ab- and/or CTL-defined epitopes into expression vectors (Fig. 1B). Residues 120–147 of the S protein form the group-specific, glycosylated, immunodominant a determinant (2) that forms a loop between two transmembrane domains (18). This determinant and the two transmembrane flanking regions are encoded by the S80–180 fragment (SII) of the S protein (Fig. 1B). The Lβ-binding, antigenic S28–39 peptide of HBsAg (19) is located in the N-terminal S1–100 fragment (SI) of the S protein (Fig. 1B).

The p24 (nonglycosylated) and gp27 (glycosylated) S protein is secreted, i.e., found in supernatants of transfectants (Fig. 2A). The p39 (nonglycosylated) and gp42 (nonglycosylated) LS protein with the preS (preS1 and preS2) and S domains are expressed in cells transfected with pCI/LS DNA (Fig. 2B). The LS protein

---

**FIGURE 1.** Construction of HBV surface fragments using a novel hsp73-binding, SV40 T-Ag-derived expression system. A, Map of antigenic regions within the HBV large (LS) and small (S) surface Ag. B, Construction of HBsAg fragments. Fusion fragments consists of the N-terminal hsp73-binding unit, i.e., the SV40 CT72 fragment (cT) or the Tβ7-fragment, or of the non-hsp-binding Tβ50 fragment and C-terminal in-frame fusion sequences S1–100 (SI), S60–180 (SII), and S80–180/20–50 (SII*L'). The HSbsAg-specific sequences encoding the immunodominant Lβ-restricted S28–39 CTL epitope and the Ab-defined S120–147 a determinant are indicated.
found in lysates but not in supernatants of transfected cells, confirming that it is largely intracellular (3). No protein product was detectable in cells after stable or transient transfection of DNA-containing sequences encoding the SII (residue 80–180) fragment of HBsAg cloned into the pCI vector (data not shown).

Expression of chimeric proteins containing a T-Ag-derived, hsp73-binding N terminus fused in-frame to fragments of the HBsAg

The SI or SII fragments of HBsAg were fused in-frame behind the hsp73-binding, N-terminal 272-residue fragment of the SV40 T-Ag from which the NLS (residue 110–152) was deleted (Fig. 1B) (14). This generated vectors encoding either the cT272-SI (pCI/cT-SI; it contains the Ld-restricted S28–39 CTL epitope, but not the Ab-defined determinant of HBsAg) or the cT272-SII fusion proteins (pCI/cT-SII; it contains the a determinant of HBsAg, but not the Ld-restricted S28–39 CTL epitope) (Fig. 1B). Products of the expected size were immunoprecipitated with the anti-T-Ag mAb PAb108 from lysates of radiolabeled, transiently transfected chicken hepatoma LMH cells (Fig. 2C). A 38-kDa fusion protein was expressed by plasmid pCI/cT-SI, and two fusion products of 38 (cT-SII) and 40 (gp cT-SII) kDa were expressed by plasmid pCI/cT-SII. Both cT-SI and cT-SII proteins were expressed at comparable levels and coprecipitated large amounts of hsp73 (Fig. 2C). We cloned the sequences encoding the cT-SI and cT-SII fusion proteins into the episomal BMGneo vector (13). With these BMG/cT-SI and BMG/cT-SII vectors, we generated stable CHO transfectants. From lysates of stable CHO/cT-SI or CHO/cT-SII transfectants, we precipitated cT-SI and cT-SII fusion proteins and coprecipitated hsp73 with anti-T-Ag mAb PAb108 that were readily detected in Coomassie blue-stained gels (Fig. 2D). Thus, high levels of fusion protein-hsp73 complexes accumulated in transfected cell lines. Western blot analyses of CHO/cT-SII immunoprecipitates with polyclonal anti-T, anti-S, and anti-hsp73 detection Abs. The positions of glycosylated and nonglycosylated forms of S, LS, cT-SI, and cT-SII and of hsp73 are indicated. Furthermore, the position of the H chain (HC) of mAb PAb108 used for immunoprecipitation is indicated (+).

Expression of the native, glycosylated a determinant of HBsAg by the fusion protein cT-SII

The conformational HBsAg a determinant is a loop between the second and third transmembrane domain of this viral envelope protein and contains a glycosylation site at Asn146 (2). Similar to the a loop in native HBsAg, the a loop in the cT-SII (S80–180) fusion protein is glycosylated. Treatment of the cT-SII (S80–180) fusion protein with the Endo H strikingly reduces the 40-kDa band at the expense of the 38-kDa band (Fig. 3D). The cT-SII fusion protein thus seems to be anchored into the membranes of the endoplasmic reticulum (ER) or a cis-Golgi compartment by its transmembrane

FIGURE 2. Expression of wild-type HBV surface Ags or chimeric, hsp73-bound fusion Ags.

Chicken hepatoma cells (LMH) were transiently transfected with pCI/S (A), pCI/LS (B), or pCI/cT-SI and pCI/cT-SII (C). Cells were labeled with [35S]methionine. Cell culture supernatants (sup) or cell lysates (lys) were immunoprecipitated with a polyclonal anti-HBsAg serum (A and B) or with the anti-T-Ag mAb PAb108 (C) and processed for SDS-PAGE followed by fluorography of the gels. Stable transfectants CHO/cT-SI and CHO/cT-SII cells were extracted and immunoprecipitated with PAb108 and processed for SDS-PAGE followed by Coomassie blue staining of the gels (D) or by Western blotting (E) using anti-T, anti-S, and anti-hsp73 detection Abs. The positions of glycosylated and nonglycosylated forms of S, LS, cT-SI, and cT-SII and of hsp73 are indicated. Furthermore, the position of the H chain (HC) of mAb PAb108 used for immunoprecipitation is indicated (+).
domains and remains stably associated within the cells that produce it (Fig. 3C). The cT-SII fusion protein is thus apparently expressed as a membrane protein in the ER containing a glycosylated, native a determinant. We found that glycosylated 40-kDa cT-SII protein accumulated preferentially in stable transfectants (Figs. 2D and 3A). hsp73 that efficiently binds the cT-SII fusion protein is expected to be located at the cytoplasmic side (Fig. 3F).

We found no evidence that hsp proteins resident in the ER (i.e., the grp78 Bip protein or hsp proteins of the hsp90 family) bind to the cT-SII fusion protein (Fig. 3B). Secretion of cT-SII is inhibited by hsp73 binding to the T-Ag-derived N terminus

Similar to native LS (2), the cT-SII protein is not released into the supernatant (Figs. 2B and 3C). Binding of hsp73 to cT-272 depends on the (DnaJ homologous) J domain in the N-terminal 77 residues of the T-Ag (21, 22). We designed two constructs in which the S80–180 (SII) fragment was fused either to the hsp73-binding 77-residue T77 N terminus or to the non-hsp-binding 60-residue T60 N terminus (Fig. 1B). The loop-forming J domain is disrupted in the T60 fragment (21, 22) and stable hsp73 binding is lost (Fig. 3E). Both fusion proteins were expressed by LMH cells transiently transfected with pCI/T77–SII or pCI/T60–SII vector DNA (Fig. 3E). In contrast to the T77-SII protein, T60-SII protein was not associated with hsp73 and was released into the supernatant by transfectants (Fig. 3E). This allowed us to compare the immunogenicity of hsp73-associated, intracellular to non-hsp-associated, secreted variant of a viral, Ab-binding envelope protein domain.

Stimulating HBsAg-specific Ab responses by DNA vaccines encoding variants of the SII domain

We compared Ab responses induced in mice by DNA vaccines encoding either native (secreted) small S, or (nonsecreted) large LS, or the a determinant-containing SII fragment of HBsAg fused to either the hsp73-associated T77 or the non-hsp73-associated T60 domain (Fig. 4A). As a control, mice were immunized with 1/100th commercial HBvax vaccine (containing rHBsAg adsorbed to aluminum hydroxide). The commercial ELISA (IMX AUSAB System; Abbott Laboratories) used detects only serum Abs recognizing the conformational a determinant of native HBsAg. The pCI/T60–SII DNA vaccine encoding the secreted, non-hsp-binding S80-180 Ag (group 2) was as efficient as the pCI/S DNA vaccine encoding native HBsAg (group 4) in stimulating a HBsAg-specific serum Ab response after i.m. inoculation of plasmid DNA. In contrast, the pCI/T77–SII DNA vaccine encoding nonsecreted, hsp73-associated S80-180 (group 3) and the pCI/LS DNA vaccine encoding a nonsecreted, natural variant of HBsAg (group 5) were two orders
DNA vaccine-elicited HBsAg antibody response

### A conformational determinants (ELISA)

<table>
<thead>
<tr>
<th>HBsAg specific IgG serum antibodies</th>
<th>HBsAg specific subtype antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCI/S</td>
<td>2 pCI/T60-SII</td>
</tr>
</tbody>
</table>

**FIGURE 4.** Priming HBsAg-specific serum Ab responses. BALB/c mice were vaccinated twice (day 0 and day 21) i.m. with 100 μg/mouse DNA of the plasmids pCI/SII (group 1), pCI/T60-SII (group 2), pCI/T77-SII (group 3), pCDS (group 4), and pCI/LS (group 5) or with 1 μg/mouse HBsAg adjuvanted in aluminum hydroxide (group 6). Sera were obtained 1 wk after the boost injection and analyzed for HBsAg-specific Abs (mIU/ml) by the commercial ELISA (IMX USAB System; Abbott Laboratories), by a subtype (IgG1 and IgG2a) ELISA using recombinant HBsAg detection Ag (A), or by Western blotting (B) using recombinant, denatured HBsAg (upper panel) or a hsp73-associated cT-preS Ag (containing the cT272 fragment and the 163-residue preS sequence (7) (lower panel). Mean serum Ab titers and their mean IgG1:IgG2a ratios of three mice per group are shown. All sera tested showed no cross-reactivity against the hsp73 autoantigen (lower panel).

of magnitude less potent in stimulating an HBsAg-specific Ab response. The pCI/SII vector DNA encoding the S80–180 fragment of HBsAg (Fig. 1B) did not express detectable amounts of the protein and did not prime a humoral anti-HBsAg response (group 1). The secreted T60-SII fusion Ag delivered by a DNA vaccine can thus prime an Ab response to a conformational HBsAg determinant as efficiently as a DNA vaccine encoding native HBsAg.

DNA-based immunization of mice with vectors encoding secreted (groups 2 and 4) and intracellular (groups 3 and 5) HBsAg variants induced high titers of HBsAg-specific serum IgG2a Abs (Fig. 4A). The IgG1:IgG2a ratios of serum Ab responses were always <0.5 (Fig. 4A). CD4+ T cells primed by vaccination with the respective expression constructs released high levels of IFN-γ upon restimulation with HBsAg (data not shown; Ref. 24), indicating that Th1-biased immune responses were induced. In contrast, injection of rHBsAg mixed with aluminum hydroxide induced high titers of HBsAg-specific serum Abs that were predominantly of the IgG1 subclass (IgG1:IgG2a ratio, >60; Fig. 4A, group 6). This Th2 bias in the immune response was also apparent in the specific cytokine profile of primed CD4+ T cells upon restimulation with HBsAg (data not shown).

In sera from mice immunized with the pCI/T77-SII, pCI/T60-SII, or pCI/S DNA vaccine, we searched in Western blot analyses for Abs binding linear HBsAg determinants using denatured rHBsAg as the detection Ag. In contrast to the efficient priming of Ab responses to the conformational a determinant of HBsAg by some of the HBsAg-encoding DNA vaccines tested (Fig. 4B), Abs binding linear HBsAg determinants were very low or undetectable (Fig. 4B). Sera from mice immunized with commercial HBvax (HBsAg/alum) developed significant levels of Abs against linear
HBsAg epitopes (Fig. 4B). Abs binding T-Ag were readily apparent in Western blot analyses using sera from pCI/cT-immunized mice and the hsp73-associated CT272-preS fusion protein as detection Ag (Fig. 4B), confirming our previous report (7). No T-Ag-binding Abs were primed by DNA vaccines encoding the T60 and T77-containing fusion constructs (Fig. 4B). Furthermore, we did not detect serum Abs binding the hsp73 autoantigen (Fig. 4B; Ref. 7). The expression of the S0–180 fragment as a fusion protein by the pCI/T60–SII and pCI/T77–SII DNA vaccines thus selectively presents the conformational determinant of HBsAg to the immune system but does not elicit Ab responses to the T-Ag-derived carrier or the hsp autoantigen.

Abs elicited by pCI/T60–SII or pCI/T77–SII DNA vaccines suppress the HBsAg antigenemia of HBs-tg mice

HBs-tg mice express high levels of liver-derived HBsAg particles in the serum (23, 24). Adoptive transfer of HBsAg-specific serum Abs into HBs-tg hosts transiently suppresses HBsAg antigenemia (24, 25). To directly compare suppression of HBsAg antigenemia in HBs-tg mice, we transferred similar serum Ab amounts from T60–SII- or T77–SII-vaccinated B6 mice, i.e., a single injection of antisera containing 100 mIU from T77–SII-immune mice, or 100 mIU from T60–SII-immune mice were injected into HBs-tg mice. The antigenemia of the transgenic host was transiently suppressed by antisera derived from mice immunized with the pCI/T60–SII or pCI/T77–SII (but not pCI/SII) DNA vaccine (Fig. 5A). The Abs primed by the S0–180 fragment of HBsAg can thus clear native HBsAg particles produced in a mouse. Suppression of HBsAg antigenemia was similar with the pCI/T60–SII- or pCI/T77–SII-induced serum Abs, suggesting that the Abs generated by the two different constructs exhibit very similar affinities. Using 50–200-mIU Ab titers for passive therapy, serum HBsAg reappeared between 2 and 4 days (Fig. 5A). Similar results were observed when different IgG, IgG1, or IgG2a Abs specific for HBsAg were injected into HBs-tg mice (24, 25).

Adoptive transfer of splenocytes from pCI/T60–SII- or pCI/T77–SII (but not the pCI/SII)-vaccinated B6 donor mice into congenic HBs-tg hosts established a long-lasting and rising Ab response to HBsAg with stable suppression of antigenemia (Fig. 5B). The induction of HBsAg-specific Abs in HBs-tg host mice was significantly higher when T60–SII-immune cells (rather than T77–SII-immune cells) were used for adoptive transfer (Fig. 5B). Adoptive transfer of splenic immune cells from pCI/T60–SII- or pCI/T77–SII-vaccinated mice into HBs-tg hosts induced suppression of antigenemia that was stable for many months posttransplantation (data not shown). Similar results were obtained in immune cell transfers using spleen cells from mice immunized with DNA- or protein-based vaccines (24, 25). The results show that the S0–180-induced Abs are biologically functional in vivo by efficiently clearing antigenemia, irrespective whether the Ag was expressed intracellular and hsp73-associated or as a secreted Ag.

Copriming humoral and CTL immunity to HBsAg using DNA vaccines that encode immunodominant, Ab- and CTL-defined determinants

We stimulated Ab- (Fig. 4A) but not CTL-mediated immunity to HBsAg by the T77–SII DNA vaccine (Fig. 6B). To add an immunodominant, L3-binding CTL epitope (S20–39) of HBsAg to the pCI/T77–SII DNA vaccine, we fused the S20–39 sequence to the C terminus of the pCI/T77–SII plasmid to generate the pCI/T77–SII-L3 construct (Fig. 1B). Similar to the T77–SII construct, two (glycosylated and nonglycosylated) T77–SII-L3 fusion proteins of the expected size were expressed by cells transfected with DNA of this construct (Fig. 6A). The T77–SII-L3 was associated with the hsp73 (Fig. 6A). The presence of the CTL-defined HBsAg epitope was confirmed in BALB/c mice injected with the pCI/T77–SII-L3 DNA (Fig. 6B). HBsAg-specific CTL reactivity was efficiently primed in mice injected with either the HBsAg-encoding pCS plasmid or the pCI/T77–SII plasmid encoding the C-terminal HBsAg 1–100 fragment, or the pCI/T77–SII-L3 plasmid (i.e., vector constructs that contained the HBsAg-specific CTL epitope(s) (see Fig. 1B). In the latter construct, the L3-restricted CTL epitope of HBsAg is expressed in an artificial position (see Fig. 1B). No HBsAg-specific CTL reactivity was primed by injecting pCI/SII or pCI/T77–SII DNA vaccines (Fig. 6B).

Coexpression of the S20–39 fragment enhanced the immunogenicity of a concomitant B cell response (Fig. 6C). HBsAg-specific Ab titers were reproducibly 2- to 3-fold higher when mice were primed by the pCI/T77–SII-L3 as compared with the pCI/T77–SII construct. However, the T77–SII-L3-primed Ab titers to HBsAg were in the range expected for intracellular HBsAg variants (Fig. 4A). pCI/T77–SII- and pCI/T77–SII-L3-immunized mice induced a comparable Th1-biased immunity to HBsAg (Fig. 4A and data not shown). In this system, CTL priming is thus not affected by

Transfer of HBV-′a′′′′′′′′′′ immune responses into HBs-tg mice

![FIGURE 5](http://www.jimmunol.org/DownloadedFromhttp://www.jimmunol.org/) Transfer of a determinant-specific serum Abs (A) and immune cells (B) establishes HBsAg immunity in HBs-tg hosts. Syngeneic C57BL/6 (B6) mice were vaccinated twice (day 0 and day 28) i.m. with 100 μg/mouse plasmid DNA of the vectors pCI/T60–SII, pCI/T77–SII, or pCI/SII. A. Serum Abs were determined 2–6 wk after the boost injection and 200–400 μl serum pooled from three to five mice was injected into host HBs-tg mice: the sera contained 100 mIU/ml (pCI/T60–SII; these sera were diluted in PBS), 100 mIU/ml (pCI/T77–SII), and no HBsAg-specific Abs (pCI/SII). B. Alternatively, we injected 3 × 10⁷ spleen cells from individual donor mice i.p. into HBs-tg mice. At the indicated time points before and after transfer, we quantitatively determined serum HBsAg levels using the AXSYM HBsAg V2 kit (A and B) and HBsAg specific serum Abs (B).

Downloaded from http://www.jimmunol.org/ by guest on April 17, 2017
copriming CD4+ T cell-dependent humoral immunity, but priming humoral immunity is facilitated by copriming CTL.

We also constructed the vector pCI/T60-SII-Ld. The data obtained with this construct are difficult to interpret. The T60-SII-L4 Ag is not associated with hsp73 and is glycosylated. Immunization of BALB/c mice with this construct induces HBsAg28-39-specific, Ld-restricted CTL responses, but no HBsAg-specific serum Ab response. The C-terminal S20-S50 sequence of the T60-SII-L4 fusion Ag might inhibit priming of humoral immunity by interfering, for example, with the proper folding of the conformational loop structure or the membrane anchoring of the S80-L180 (SII) fragment.

Discussion

To select immunodominant B and T cell-defined determinants from the same or different Ags of a pathogen, or from different pathogens, coexpress them in a construct and codeliver them in an immunogenic way as a polyvalent vaccine are long-standing goals in vaccinology. Major problems in this approach are 1) the expression of conformational, Ab-binding epitopes; 2) limits in the amount of immunogenic information that can be incorporated into a single vaccine; and 3) specific negative regulatory events that can prevent efficient copriming of T and B cell responses to a single recombinant Ag. The system we describe supports 1) selective expression of a conformational B cell epitope in immunogenic form, 2) stable expression of large constructs as hsp73-associated fusion proteins, and 3) copriming of CTL and B cell responses to different determinants.

In DNA-based vaccination, plasmid DNA-containing Ag-encoding sequences and appropriate promoter/enhancer control sequences are injected into muscle or skin. This leads to expression of the Ag and its immunogenic presentation by in vivo-transfected cells. The “vaccine” in nucleic acid immunization is usually plasmid DNA, although successful vaccination with Ag-encoding mRNA has been reported (26, 27). Many aspects of DNA-based vaccination have been explored extensively (28, 29). The introduction of DNA vaccines has opened new dimensions for expressing and testing recombinant Ags produced in a large variety of novel systems. Some systems support the construction of large polyepitope vaccines, the incorporation of “intrinsic adjuvant activity” into vaccines, and/or the targeting of vaccines to distinct tissues or cells.

Mice immunized into the tibialis anterior muscles with 100 μg of the small HBsAg-encoding pCI/S plasmid DNA developed high Ab titers specific for HBsAg (Fig. 4A). Abs appeared in the third week postvaccination, reached plateau levels at 16–20 wk postvaccination, and persisted at high levels for >9 mo (30). Similar data have been described using different HBsAg expression plasmids (31–34). Intrinsic properties of the Ag might have a decisive influence on the type of specific immunity that is primed by DNA vaccination. It has been reported that the nature of the (intracellular or secreted) Ag expressed by a DNA vaccine can affect the levels of induced Abs (35, 36). It is unclear how DNA vaccines prime Ab responses to intracellular protein Ags as it is usually assumed that B cells require exogenous Ag to be stimulated. As the pCI/S vector expressed secreted HBsAg, exogenous Ag can directly prime Ab responses in this system. We showed that mice immunized with pCI/LS DNA encoding the nonsecreted (intracellular) LS variant of HBsAg developed low HBsAg-specific Ab titers (Fig. 4B; (30, 37). It is not clear how B cells are primed by intracellular Ags. It is generally assumed that small amounts of intracellular Ags are released from the cells that synthesize the Ag. This process might be supported by cell death induced by Ag-specific CTL (29).

We developed an intracellular expression system based on the observation of an unexpected accumulation of large amounts of mutant SV40 T-Ag with a long half-life in transfectants and a tight ATP-dependent binding of hsp73 to mutant but not wild-type T-Ag (12, 20). We designed fusion constructs with an N-terminal, hsp73-binding domain of T-Ag and unrelated C-terminal sequences (encoding Ag fragments) that showed strikingly enhanced and stable expression (7). This expression system was also used in

![FIGURE 6. Design of a polyepitope vaccine. LMH cells were transiently transfected with pCI/Tγ-SII or pCI/Tγ-SII-L4 DNA, labeled with 131I-methionine, extracted, and immunoprecipitated with anti-T-Ag mAb Pab108 and processed for SDS-PAGE followed by fluorography of the gels (A). The positions of glycosylated and nonglycosylated forms of Tγ-SII, Tγ-SII-L4, and hsp73 are indicated. B and C, BALB/c mice were vaccinated twice (day 0 and day 28) i.m. with 100 μg plasmid DNA of the indicated vectors: pCI/Tγ-SII-Ld (group 1), pCI/Tγ-SII (group 2), pCI/SII (group 3), pCI/T-SI (group 4), and pCI/S (group 5). Spleen cells obtained 11 days after vaccination were restimulated in vitro for 5 days with HBsAg-expressing P815 transfectants and tested for specific cytotoxicity in a 4-h 51Cr release assay. Mean specific lysis values of P815/S transfectants (of triplicates) at the indicated E:T ratios are shown. The nonspecific lysis of control P815 (≤5%) was subtracted (B). Sera from pCI/Tγ-SII-Ld (group 1), pCI/Tγ-SII (group 2), and pCI/SII (group 3)–immunized mice were obtained 2–3 wk after the boost injection and analyzed for HBsAg-specific Abs (mIU/ml) in ELISA (C). Mean Ab titers of three to six mice per group are shown.](http://www.jimmunol.org/)

Downloaded from http://www.jimmunol.org/ by guest on April 17, 2017
these studies to efficiently and selectively express B and T cell epitopes of HBsAg. Some aspects of the biology of the system have been elucidated. The N terminus of the T-Ag of papovaviruses contains the highly conserved J domain (i.e., sequences homologous to the Escherichia coli DnaJ molecule) with a conserved HDP loop that recruits cellular chaperones, e.g., the cytosolic hsp73 chaperone (8, 21, 22). J domains contain four α helices with helix II and III forming a finger-like loop structure that exposes the conserved HDP motif at the tip. Mutations in the J domain disrupt its helical structure and prevent its association with hsp73 (21). We confirmed that an intact N terminus of T-Ag containing the J domain is required for hsp73 association as the T77 fragment, but not the T60 (with a disrupted DnaJ homologous structure) binds to hsp73. By constructing fusion Ags containing the same C-terminal antigenic determinant fused to hsp-binding (T77) or hsp-nonbinding (T60) N terminus, we could test the pattern of expression and compare the immunogenicity of the recombinant Ags. The secreted T60-SII construct was not associated with hsp. The T77-SII construct was hsp-associated and expressed intracellularly. Both fusion Ags were expressed as nonglycosylated and glycosylated species, suggesting correct transmembrane incorporation of the a loop containing the S80-180 fragment (Fig. 3F) (18, 38). The secretion-defective, hsp73-associated T5-SII protein preferentially accumulates in transfected cells in its glycosylated form (Figs. 2Dand 3A). The glycosylated T77-SII protein was sensitive to treatment with Endo H (Fig. 3D), suggesting that this protein accumulates in the ER or cis-Golgi structures and was not transported to the medial cisternae of the Golgi apparatus where carbohydrate chains acquire resistance to this enzyme (17). In contrast, significantly reduced levels of the T60-SII protein (not associated with hsp73) were expressed and secreted by transfected cell lines (Fig. 3E). The glycosylated T60-SII form was barely detectable (Fig. 3E) and the T60-SII protein did not accumulate to detectable steady-state levels in stable transfected cell lines (data not shown). Secretion of the S60-180 encoding T60-SII protein was unexpected as similar HBsAg fragments with deletions in the first hydrophilic region of HBsAg (e.g., the S83-226) remain intracellular (17, 39, 40).

The pCI/SII vector DNA encoding the 80- to 180-residue fragment of HBsAg with a synthetic ATG at its N terminus did not express detectable amounts of the protein, and did not prime a humoral anti-HBsAg response. In contrast, the SII fragment was expressed when fused to the 60-residue T-Ag (T60-SII protein). We tested alternative ways to express this fragment. The SII (S60-180) fragment of HBsAg was fused in-frame behind the N-terminal 5- or 45-residue fragment of the T-Ag or in-frame to an ATG containing pcDNA4/HisMax-C (Invitrogen, Karlsruhe, Germany) and was expressed to similar levels and molecular characteristics (secreted and glycosylated) as the T60-SII protein (data not shown).

As expected for secreted Ags, comparable HBsAg-specific serum Ab responses were stimulated after inoculation of the pCI/T5-SII, pCI/T4.5-SII, pcDNA4/HisMax-SII, and pCI/T60-SII DNA vaccines (data not shown). These findings suggest that the S-H fragment itself was efficiently expressed with selected ATG start codons.

The T60-SII fusion Ag primed a specific serum Ab response to the native a determinant of HBsAg when expressed from a DNA vaccine. The HBsAg-specific Ab titers induced by this secreted Ag reached comparable titers as the secreted wild-type S (Fig. 4A). As expected, the secreted T60-SII variant of the Ag was more efficient in stimulating an Ab response than the intracellular T77-SII variant of the Ag (Fig. 4A). The “innate adjuvant effect” of hsp73 molecules that facilitates CTL priming (41) does apparently not operate for priming B cells to the a determinant of HBsAg. However, it does facilitate priming of Ab responses to the preS domain of HBsAg (7) or to HBV precore/core Ag domains (R. Schirmbeck, unpublished data). Hence, we conclude that expression of the a determinant is sufficient to induce efficient immune responses. The low Ab response elicited by intracellular T77-SII Ag was biologically active because it strikingly reduced the HBsAg antigenemia in HBs-tg mice. This observation is central to our postulate that biologically functional Abs (cross-)reactive to a conformational determinant of the native viral Ag are generated in the system. It is accepted that a conformationally intact a determinant of HBsAg is required for neutralization. The glycosylated HBsAg particles produced in the liver of HBs-tg mice contain LS, MS, and S and elicit a-specific Ab responses when injected into naive hosts (42). The transient suppression of antigenemia after serum transfer and the stable suppression of antigenemia after immune cell transfers suggest that potentially neutralizing Ab responses were elicited by the vaccine.

Fusing a CTL-defined epitope to the recombinant Ag enhanced the Ab response. This may help to open up Ag-bearing cells in vivo and thereby facilitate recognition of intracellular Ag by B cells. Coprimering B cell and CTL immunity can be suppressive or provide help. CTL can selectively kill specific B cells (43, 44) or B cells can tolerate CTL (45–47). B cells can present Ag to CTL (48–50) but may preferentially activate Ag-experienced rather than naive T cells (51). Our data provide no evidence for inhibition in coprimering B cell and CTL immunity to HBsAg, although both determinants are expected to be presented by the same APC. This is encouraging for the prospects of a polyepitope vaccine containing Ab- and T cell-stimulating determinants.

We have demonstrated the feasibility to 1) selectively express a conformational, Ab-stimulating epitope in a DNA vaccine; 2) model the system in a way that resembles the expression and immunogenicity of two natural variants of the Ag of interest (i.e., the secreted S particle and the intracellular LS protein); and 3) integrate B cell- and CTL-stimulating epitopes into a single polyepitope construct. The system revealed some features that may help in the design of new generations of polyvalent, DNA- or protein-based vaccines.

Acknowledgments
The excellent technical assistance of Tanja Guentert and Claude Oehninger is appreciated. The generous gift of recombinant HBsAg from Dr. K. Meller (Rhein Biotech, Dusseldorf, Germany) is gratefully acknowledged.

References


