Hepatitis B Surface Antigen Vector Delivers Protective Cytotoxic T-Lymphocyte Responses to Disease-Relevant Foreign Epitopes†

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Although hepatitis B surface antigen (HBsAg) per se is highly immunogenic, its use as a vector for the delivery of foreign cytotoxic T-lymphocyte (CTL) epitopes has met with little success because of constraints on HBsAg stability and secretion imposed by the insertion of foreign sequence into critical hydrophobic/amphipathic regions. Using a strategy entailing deletion of DNA encoding HBsAg-specific CTL epitopes and replacement with DNA encoding foreign CTL epitopes, we have derived chimeric HBsAg DNA immunogens which elicited effector and memory CTL responses in vitro, and pathogen- and tumor-protective responses in vivo, when the chimeric HBsAg DNAs were used to immunize mice. We further show that HBsAg DNA recombinant for both respiratory syncytial virus and human papillomavirus CTL epitopes elicited simultaneous responses to both pathogens. These data demonstrate the efficacy of HBsAg DNA as a vector for the delivery of disease-relevant protective CTL responses. They also suggest the applicability of the approach of deriving chimeric HBsAg DNA immunogens simultaneously encoding protective CTL epitopes for multiple diseases. The DNAs we tested formed chimeric HBsAg virus-like particles (VLPs). Thus, our results have implications for the development of vaccination strategies using either chimeric HBsAg DNA or VLP vaccines. HBsAg is the globally administered vaccine for hepatitis B virus infection, inviting its usage as a vector for the delivery of immunogens from other diseases.

Vaccination strategies capable of safely and effectively inducing cytotoxic T-lymphocyte (CTL) responses which are acceptable in humans have not proven easy to develop (18). The hepatitis B virus (HBV) surface antigen (HBsAg) small envelope protein (HBsAg-S) self-assembles into highly organized virus-like particles (VLPs) (11). HBsAg VLPs are extremely efficient at inducing long-lived HBsAg-specific CTL responses when administered as a vaccine, comparing favorably with traditional strong CTL inducers in this regard (40). This likely relates to the presentation of the CTL epitopes as multiple copies in repetitive arrays, a format known to enhance induction of CTL responses (21). HBsAg may also be delivered as a DNA vaccine. By use of this strategy, CTL induction by intracellular translated HBsAg protein occurs through the classical endogenous pathway as well as through the alternative exogenous pathway via secreted HBsAg particles (28, 38, 39).

HBsAg-S polypeptide is composed of 226 residues, is synthesized at the endoplasmic reticulum anchored at the lipid membrane and is secreted as empty 22-nm VLPs, with each VLP consisting of 100 to 150 polypeptides. The HBsAg molecule has a complex configuration and contains at least two transmembrane regions, an internal luminal domain and a hydrophilic external domain (the a determinant). The C-terminal domain is hydrophobic and it probably contains two additional transmembrane regions. HBsAg CTL epitopes are

located primarily in the transmembrane and luminal domains (2, 42).

Although HBsAg per se is highly immunogenic, the use of chimeric HBsAg to deliver foreign epitopes has met with limited success (particularly in the delivery of CTL epitopes), probably because the strategy hitherto used, that of introducing foreign sequences into the HBV genome at sites where restriction endonuclease sites fortuitously exist or of extending the termini of HBsAg protein with epitope sequences, does not necessarily account for spatial requirements for VLP formation (2, 11). In the present study, we report a novel strategy to exploit the superior immunogenicity of HBsAg by deleting HBsAg-specific CTL epitopes and replacing them with foreign CTL epitopes of similar physical properties (i.e., size and hydrophobicity). We reasoned this approach might predispose to the maintenance of spatial requirements, the conformation of the HBsAg molecule, and, in addition, the maintenance of appropriate antigen processing.

We demonstrate that immunization with DNA encoding chimeric HBsAg in which an HBsAg-specific CTL epitope(s) was deleted and replaced with CTL epitopes of respiratory syncytial virus (RSV) or human papillomavirus (HPV) elicited strong CTL responses to the inserted epitopes. Immunization with chimeric HBsAg DNA afforded protection against RSV infection or the growth of HPV type 16 (HPV16)-associated tumors. We further show that immunization with DNA encoding HBsAg recombinant for both RSV and HPV CTL epitopes elicited CTL responses to both foreign epitopes and afforded protection against disease.

We demonstrate that chimeric HBsAg DNAs constructed using the deletion-replacement strategy form VLPs which are secreted from cells. HBsAg VLPs are globally licensed as a

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TABLE 1. Epitopes used in the study

Epitope or mimotope	Protein	Residue	Sequence	Reference
CTL epitopes				
IPQ	HBsAg	28–39 ^a	IPOSLSWWTSL	42
GLS	HBsAg	185–194 ^a	GLSPTVWLSV	28
FLL	HBsAg	20–28 ^a	FLLTRILTI	28
M2 ₉	RSV M2	82–90	SYIGSINNI	22
M2 ₁₅	RSV M2	81–95	ESYIGSINNITKQSA	22
RAH	HPV16 E7	49–57	RAHYNIVTF	14
B mimotope				
HW1	RSV F	NA ^b	HWSISKPO	4

^a In HBsAg-S.^b NA, not applicable.

vaccine for HBV infections in humans (including children) with a ca. 12-year history of use in millions of recipients, inviting their usage as a vector for the delivery of immunogens from other diseases. Our results demonstrate that the powerful immunogenicity of HBsAg may be exploited to deliver foreign CTL epitopes relevant for other diseases and have generic implications for the development of HBsAg as a multivalent vaccine vector. There are also specific implications for vaccines against RSV infection and HPV-associated carcinoma.

MATERIALS AND METHODS

Cloning procedures. The plasmid pcD3-HBsAgS, which contains a AgeI restriction site at a location corresponding to amino acids (aa) 127 and 128 of the a determinant region (33), was engineered to delete the HBsAg-specific CTL epitopes IPQ and GLS (Table 1) and to introduce the restriction enzyme sites NheI and BspI, respectively, by PCR-driven site-directed mutagenesis. Plasmid pM2₁₅ΔIPQ (Table 2) was derived by inserting DNA encoding the RSV-specific M2₁₅ epitope at the NheI site. Further chimeric HBsAg plasmids were derived as summarized in Table 2. Plasmid pM2₁₅ΔIPQ.RAHΔGLS.HW1 was derived by inserting DNA sequences encoding the RSV B-cell-specific mimotope (4) into the AgeI restriction site and then sequentially inserting DNA encoding the M2₁₅ epitope and the RAH epitope at the ΔIPQ and ΔGLS sites, respectively. Standard molecular cloning and plasmid purification procedures were used. All plasmids were verified by DNA sequencing (PCR Sprint temperature cyclin system [Hybaid]).

Transfection. HuH-7 cells (31) were transfected with bacterial plasmid DNA by use of calcium phosphate. The supernatant was harvested 5 days later, and HBsAg was measured by the Abbott Prism HBsAg assay (Abbott Diagnostics). The level of HBsAg in the cell culture fluid was quantified by comparison with a commercially available vaccine (Engerix-B, 20 µg/ml; Smith-Kline Beecham). The transfection efficiencies of different plasmids were normalized to the activity of secreted alkaline phosphatase as previously described (1). The variation in the

range of secreted alkaline phosphatase was less than twofold. The presence of hepatitis delta virus (HDV) large antigen (HDAg-L) was identified by immunoblotting using a human anti-HDV antibody and assayed by the ECL-plus detection system (Amersham) (33).

Mice. A2.1K^b mice (44) make CTL responses restricted through HLA A*0201 and H-2^b class I molecules. (A2.1K^b × BALB/c)F₁ hybrid mice additionally make CTL responses restricted through H-2^d. Mice were housed under specific-pathogen-free conditions and used at 7 to 15 weeks of age.

Peptides. Peptides were synthesized using 9-fluorenylmethoxy carbonyl chemistry and analyzed by high-performance liquid chromatography and by amino acid analysis by Chiron Mimotopes (Melbourne, Australia). The RSV F protein mimotope was synthesized as a tetrameric multiple antigen peptide construct as described previously (4).

Immunization and restimulation of splenocytes. Mice were immunized twice at 2-week intervals intradermally (i.d.) in the ear with 100 µg of purified plasmid DNA. Two weeks later, spleens were removed and splenocytes were restimulated in vitro for 6 days as described previously (12) with 1 µg/ml cognate peptide. For peptide immunizations, mice were immunized subcutaneously (s.c.) at the tail base with 50 µg peptide plus 0.25 µg tetanus toxoid as a source of T-helper epitopes plus 10 µg Quil A adjuvant (24). Ten days later, spleens were harvested and splenocytes were restimulated as described above.

Cells. EL4.A2 cells (12) are susceptible to specific CTL lysis through both H-2^b and HLA A*0201 restriction pathways. P815 is susceptible to specific CTL lysis through the H-2^d restriction pathway. Cells were maintained as described previously (12).

CTL assays. CTL assays were conducted as previously described (12). In summary, target cells (10⁴ per well), sensitized at 37°C for 1 h with 1 µg/ml cognate or irrelevant peptide or with medium alone and labeled with 100 µCi ⁵¹chromium (Cr), were incubated with effector cells at various effector-to-target cell ratios in triplicate in 96-well microtiter plates. Negative controls included wells containing target cells but no effector cells (background). Supernatants were harvested from CTL assays at 4 h, and ⁵¹Cr release was quantified by gamma counting. Results are expressed as percent cytotoxicity ± standard deviation [(⁵¹Cr release in experimental wells – background/detergent-mediated total release – background) × 100%]. Experimental and control values were compared for significant difference using Student's *t* test.

Murine IFN-γ ELISPOT assay. Epitope-specific gamma interferon (IFN-γ)-secreting spleen cells were enumerated ex vivo by an enzyme-linked immunospot (ELISPOT) assay with minimal CD8⁺ T-cell epitope peptides essentially as described previously (24). IFN-γ spots were counted using an AID ELiSpot reader system. Results were calculated as IFN-γ-positive cells/10⁶ spleen cells. Experimental and control values were compared for significant difference using analysis of variance.

RSV propagation. HEP-2 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Invitrogen, Mt. Waverley, VIC, Australia) supplemented with 292 µg/ml GlutaMax-1 (Gibco BRL), 1 mM sodium pyruvate, 20 mM HEPES, 50 µM β-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco BRL), and 10% fetal bovine serum (CSL Ltd, Parkville, Australia). RSV A2 (VR-1302) strain (American Type Culture Collection) was adsorbed onto the HEP-2 cell monolayer at a multiplicity of infection of 0.04 PFU/cell for 2 h at 37°C prior to culturing in supplemented RPMI 1640 medium for 3 to 6 days. RSV was recovered from the supernatant following

TABLE 2. Summary of constructs in which HBsAg-specific CTL epitopes are replaced with foreign CTL epitopes

Deleted HBsAg CTL epitope(s) (size)	MHC restriction	Replacement foreign CTL epitope(s) (size)	MHC restriction	Net size change(s) (aa) ^a	B-epitope insertion into the a loop	Construct name
IPQ (12-mer)	H-2L ^d	M2 ₉ (9-mer)	H-2K ^d	0		pM2 ₉ ΔIPQ
IPQ (12-mer)	H-2L ^d	M2 ₁₅ (15-mer)	H-2K ^d	+6		pM2 ₁₅ ΔIPQ
IPQ (12-mer)	H-2L ^d	RAH (9-mer)	H-2D ^b	0		pRAHΔIPQ
GLS (9-mer)	HLA A*0201	M2 ₁₅ (15-mer)	H-2K ^d	+9		pM2 ₁₅ ΔGLS
GLS (9-mer)	HLA A*0201	RAH (9-mer)	H-2D ^b	0		pRAHΔGLS
IPQ, GLS	H-2L ^d , HLA A*0201	M2 ₁₅ , M2 ₁₅	H-2K ^d	+6, +9	HWI	pM2 ₁₅ ΔIPQ.ΔGLS.HW1 ^b
IPQ, GLS	H-2L ^d , HLA A*0201	M2 ₁₅ , RAH	H-2K ^d and H-2D ^b	+6, 0		pM2 ₁₅ ΔIPQ.RAHΔGLS
IPQ, GLS	H-2L ^d , HLA A*0201	M2 ₁₅ , RAH	H-2K ^d and H-2D ^b	+6, 0	HW1	pM2 ₁₅ ΔIPQ.RAHΔGLS.HW1 ^b

^a The net size change incurred by CTL epitope replacement is determined by the number of amino acids of the HBsAg CTL epitope deleted, the number of amino acids introduced by in-frame insertion of the RE site, and the number of amino acids the foreign epitope inserted.

^b The net size change increases by a further 8 aa of the inserted B mimotope.

FIG. 1. Immunization with chimeric HBsAg DNA encoding an RSV CTL epitope at Δ IPQ and/or Δ GLS elicits RSV-directed CTL responses. (a to e) Epitope-specific IFN- γ -secreting T-cell response following immunization of BALB/c mice (three per group) immunized twice i.d. (a) with pM2₁₅ΔIPQ, (b) with pM2₁₅ΔIPQ, or (c) with pM2₁₅ΔGLS. Control mice were immunized (d) once with M2₁₅ peptide plus adjuvant s.c. or (e) twice with wild-type HBsAg DNA (pHBsAg) i.d. IFN- γ -secreting cells were quantified by ELISPOT assay in splenocytes harvested at 14 days (DNA immunizations) or 10 days (peptide immunizations) after immunization and incubated for 15 to 18 h with specific peptide (M2₁₅ or IPQ) or without peptide, as shown. Bars represent means $\pm$ standard deviations of three replicates. (f to j) Epitope-specific CTL memory responses. Groups of mice (three per group) were immunized as described above. Percent cytotoxicity of splenocytes restimulated with RSV-specific M2₁₅ or HBsAg-specific IPQ peptide was measured in a ⁵¹Cr release assay using target cells pulsed with M2₁₅ or IPQ peptide or without peptide as shown. Data points represent means of three replicates $\pm$ standard deviations. (Note that in some cases standard deviation bars, though plotted, are too small to appear.) (k and l) BALB/c mice (three per group) were immunized three times i.d. with chimeric HBsAg DNAs encoding the M2₁₅ CTL epitope inserted either at one location (pM2₁₅ΔIPQ.HWI or pM2₁₅ΔGLS.HWI) or at two locations (pM2₁₅ΔIPQ.ΔGLS.HWI). Control mice were immunized with pHBsAg wild-type DNA. (k) IFN- γ -secreting cells were quantified by ELISPOT assay using ex vivo splenocytes incubated with or without M2₁₅ peptide. (l) Percent cytotoxicity of splenocytes restimulated for 6 days with M2₁₅ peptide was quantified in a ⁵¹Cr release assay using target cells pulsed with M2₁₅ peptide or without peptide as shown.

freeze-thawing of monolayers displaying a cytopathic effect. RSV was quantified by immunofocus assay (16), using goat anti-RSV primary antibody (Chemicon, Australia), anti-goat immunoglobulin (Ig) horseradish peroxidase-conjugated second antibody, and DAB (tetra-aminobiphenyl hydrochloride) substrate (Sigma, Australia) according to the manufacturer's instructions.

Evaluation of RSV infection. Mice were inoculated intranasally with 8×10^5 PFU of RSV and processed for histochemical staining or immunofluorescence using goat anti-RSV antibody and fluorescein isothiocyanate anti-goat Ig-detecting antibody (Chemicon). Lung samples were also used for RSV quantitation by plaque assay (16). Plaques were confirmed by immunostaining with goat anti-RSV antibody (Chemicon) followed by treatment with anti-goat Ig horseradish peroxidase-conjugated antibody (Chemicon, Australia) and development with DAB substrate (Sigma, Australia) and urea-H₂O₂-NaCl according to the manufacturer's instructions (Sigma).

Tumor protection assays. Groups of H-2^b mice (five per group) were immunized with 10 μ g plasmid DNA i.d. or with 100 μ g of E7 peptide plus tetanus

toxoid plus Quil A s.c. TC-1 cells, which express the E7 tumor-associated antigen of human papillomavirus type 16 (27), were subsequently injected (2×10^5 in 0.1 ml Hanks' buffered salt solution) s.c. on the flank. (The tumor dose was predetermined by titration experiments to discern a minimal dose giving rise to tumor in 100% of unimmunized mice.) Tumor growth was monitored every 2 days, and mice were euthanized when tumor volumes exceeded 1,000 mm³. Unimmunized mice received the same number of cells and served as a control. Data are represented as Kaplan-Meier curves of the percent tumor-free mice at given time points after tumor injection.

RESULTS

Immunization with chimeric HBsAg DNA encoding the RSV M2 CTL epitope elicits M2-directed CTL. A number of HBsAg CTL epitopes in hydrophobic regions of the HBsAg molecule,

restricted through murine (39, 42) and human (28) major histocompatibility complex class I (MHC-I) molecules, have been described previously. In a first set of experiments, we elected to delete two of the stronger of these HBsAg-specific CTL epitopes (IPQ and GLS [Table 1]) and replace them with a protective epitope from the M2 protein of RSV. The minimal M2 CTL epitope of RSV is a 9-mer represented by M2 residues 82 to 90 (designated M2₉); a larger 15-mer peptide sequence represented by residues 81 to 95 (designated M2₁₅) also functions as a CTL epitope (Table 1 and reference 20). We asked whether chimeric HBsAg DNA vaccines encoding the RSV CTL epitope would elicit M2-directed effector and memory CTL responses.

Effector responses. We quantified epitope-specific IFN- γ secretion of ex vivo splenocytes by ELISPOT assay. Ex vivo splenocytes from mice immunized with pM2₉ΔIPQ, pM2₁₅ΔIPQ, or pM2₁₅ΔGLS DNA (Table 2) secreted significant amounts of IFN- γ when cultured in vitro with, but not without, cognate M2 peptide (Fig. 1a to c), indicating the induction of an M2-directed effector CTL response. Ex vivo splenocytes from mice immunized with wild-type HBsAg DNA (pHBsAgWT) and cultured with M2 peptide did not secrete IFN- γ above the level observed when culturing was done without peptide (data not shown).

Memory response. We also asked whether immunization with pM2₉ΔIPQ, pM2₁₅ΔIPQ, or pM2₁₅ΔGLS DNA would induce memory CTL capable of being restimulated in vitro to kill target cells displaying the M2 CTL epitope. Splenocytes from pM2₉ΔIPQ, pM2₁₅ΔIPQ, or pM2₁₅ΔGLS DNA-immunized mice were restimulated in vitro for 6 days with M2₁₅ peptide and reacted with H-2^d target cells pulsed with M2₁₅ epitope peptide. Restimulated splenocytes from all these immunized groups of mice killed M2₁₅ peptide-pulsed targets (Fig. 1f to h). Notably, the efficiency of killing was comparable to that seen in identically restimulated splenocytes from M2₁₅ peptide-plus-adjuvant-immunized mice (Fig. 1i). (Immunization with high-dose CTL epitope peptide plus adjuvant is a powerful inducer of CTL responses [12] and is a standard against which other CTL-inducing modalities may be compared.) It is noteworthy that the percent killing of M2-pulsed target cells by pM2₉ΔIPQ or pM2₁₅ΔIPQ DNA-immunized mice exceeded the percent killing of target cells pulsed with the HBsAg-specific peptide IPQ by restimulated splenocytes from pHBsAgWT immunized mice (Fig. 1j). Taken together, these data indicate that replacement of DNA encoding HBsAg-specific CTL epitopes (IPQ and GLS) with DNA encoding a CTL epitope from the RSV M2 protein to generate chimeric HBsAg DNA immunogens engenders M2-directed effector and memory CTL responses of intensities comparable to those engendered by immunization with peptide plus adjuvant and to those of the HBsAg-specific epitopes engendered by immunization with pHBsAg wild-type DNA.

Chimeric HBsAg immunogen containing two copies of foreign epitope. We also asked whether two copies of the M2 epitope, inserted at the IPQ and GLS sites in HBsAg, respectively, would elicit an M2-directed CTL response that was enhanced in comparison with that elicited by chimeric HBsAg containing a single copy at the IPQ site or at the GLS site alone. Mice were immunized with a construct containing two pM2₁₅ epitopes at the different sites (pM2₁₅ΔIPQ.ΔGLS.HWI

TABLE 3. RSV infection in lungs of mice after immunization with chimeric HBsAg DNA encoding a protective CTL epitope of RSV M2 protein or with WT HBsAg

Mouse group	Immunization	No. of mice with detectable ^a RSV in lungs/total (%)	Mean RSV load in lungs ^b
1	pM2 ₁₅ ΔIPQ DNA	1/5 (20)*	$2.2 \times 10^{3**}$
2	pM2 ₁₅ ΔGLS DNA	1/5 (20)*	$2.0 \times 10^{3**}$
3	M2 ₁₅ peptide + adjuvant	1/5 (20)	1.0×10^3
4	pHBsAgWT DNA	4/5 (80)*	$8.0 \times 10^{3**}$ $6.0 \times 10^{3**}$ $7.0 \times 10^{3**}$ $7.0 \times 10^{3**}$

^a Limit of detection, 5×10^2 PFU/lung. *, $P < 0.0001$ (groups 1 and 2 versus group 4).

^b In mice with detectable infection. **, $P < 0.0001$ (groups 1 and 2 versus group 4) (analysis of variance).

[Table 2]) or with constructs containing one M2₁₅ epitope but at different sites (pM2₁₅ΔIPQ.HWI and pM2₁₅ΔGLS.HWI). (Note that the actual insertion at the two sites differed because of the introduction of different endonuclease restriction half-sites flanking the M2₁₅ epitope [Table 2 and “Cloning procedures” in Materials and Methods].) No significant augmentation of the number of IFN- γ -secreting ex vivo splenocytes (Fig. 1k) or of the cytotoxic efficacy of restimulated splenocytes (Fig. 1, panel l) compared to that seen for mice immunized with chimeric HBsAg DNA encoding one copy of the epitope was recorded for mice immunized with chimeric HBsAg DNA encoding two copies.

Immunization with plasmid encoding chimeric HBsAg containing a CTL epitope of RSV M2 protein partially protects against RSV infection. Groups of mice immunized with DNA encoding M2-containing HBsAg or wild-type HBsAg were inoculated with RSV, and 4 days later, lungs were removed for RSV quantitation, histology, and RSV-directed immunofluorescence. Four of five mice immunized with wild-type HBsAg DNA displayed detectable RSV, whereas only one of five mice immunized with pM2₁₅ΔGLS or pM2₁₅ΔIPQ DNA displayed detectable RSV in lungs (Table 3). Among those mice where RSV was detected, the viral loads per lung were significantly less in mice immunized with pM2₁₅ΔGLS or pM2₁₅ΔIPQ DNA than in mice immunized with DNA encoding wild-type HBsAg (Table 3).

The lungs of wild-type HBsAg-immunized mice challenged with RSV showed a pathology consistent with severe interstitial pneumonia (bronchiolar epithelial cell damage, extensive infiltrations of inflammatory cells, and hemorrhaging into the inflamed bronchiole) (Fig. 2a, panel i). However, lungs from mice immunized with plasmid pM2₁₅ΔGLS (Fig. 2a, panel ii) or pM2₁₅ΔIPQ (not shown) displayed less bronchiolar epithelial cell damage, little mononuclear cell infiltration of the alveoli, and less hemorrhaging into bronchioles. Peri- and extrabronchiolar RSV infection was detected in immunofluorescence-stained lung cryosections of pHBsAgWT-immunized mice (Fig. 2b, panel i) but not in those of mice immunized with pM2₁₅ΔGLS DNA (Fig. 2b, panel ii).

Together, the data in Table 3 and Fig. 2 indicate that immunization with chimeric HBsAg DNA expressing a CTL

FIG. 2. Chimeric HBsAg DNA encoding RSV M2 CTL epitope elicits protection against RSV infection and disease. BALB/c mice (five per group) were (ii) immunized twice i.d. with pM2₁₅ΔGLS DNA or (i and iii) unimmunized and (i and ii) challenged 2 weeks later with 8×10^5 PFU RSV or (iii) left unchallenged. Four days later, mice lungs were processed for (a) hematoxylin-eosin staining and (b) immunostaining with goat anti-RSV antibody.

epitope of the RSV M protein confers protection against RSV infection and associated pathology.

Immunization with plasmid encoding chimeric HBsAg containing an HPV16 E7 CTL epitope elicits E7-specific CTL responses and protects against E7-expressing tumor. We extended the concept of HBsAg-vectored delivery of protective CTL epitopes to the RAH CTL epitope of the E7 tumor-associated antigen of human papillomavirus type 16 (Table 1). We inserted DNA encoding the RAH epitope into the ΔIPQ site of the HBsAg gene to create the recombinant construct pRAHΔIPQ (Table 2). Ex vivo splenocytes from mice immunized with pRAHΔIPQ DNA, but not from those immunized with pHBsAg wild-type DNA, and cultured with RAH peptide contained a significantly higher number of IFN-γ-secreting cells than splenocytes cultured without RAH peptide (Fig. 3a and b). Additionally, restimulated splenocytes from pRAHΔIPQ DNA-immunized mice, but not from pHBsAg wild-type DNA-immunized mice, specifically lysed target cells pulsed with RAH peptide (Fig. 3d and e). These data indicate specific in vivo priming of RAH-directed effector and memory T cells by immunization with pRAHΔIPQ DNA. To determine whether immunization would protect against E7-expressing tumor, groups of mice were immunized with plasmids pRAHΔIPQ and pHBsAg and challenged with TC-1 tumor cells.

Eighty percent of mice immunized with pHBsAg developed tumor within 15 days. In contrast, none of the mice immunized with pRAHΔIPQ DNA developed tumor (Fig. 3g). The pRAHΔIPQ-induced in vitro and in vivo responses compared favorably with those induced by immunization with RAH peptide plus adjuvant (Fig. 3c, f, and h).

Together, these data indicate that immunization with DNA encoding rHBsAg containing a CTL epitope (RAH) of HPV16 E7 oncoprotein protects mice against challenge with an E7-expressing tumor, and this protection is associated with RAH-directed effector and memory CTL responses.

Immunization with a plasmid encoding chimeric HBsAg containing two protective CTL epitopes from distinct pathogens elicits CTL responses to both epitopes. We extended the concept of HBsAg-vectored protective CTL epitopes to the concomitant delivery of RSV and HPV epitopes delivered by the same chimeric HBsAg DNA. Mice were immunized with pM2₁₅ΔIPQ.RAHΔGLS.HW1 DNA (Table 2), and CTL responses were examined by IFN-γ ELISPOT assay on ex vivo splenocytes and by ⁵¹Cr release cytotoxicity assay mediated by specifically restimulated splenocytes. Splenocytes from pM2₁₅ΔIPQ.RAHΔGLS.HW1-immunized mice secreted IFN-γ when cultured with M2₁₅ peptide (Fig. 4a). The M2₁₅-directed response was similar ($P > 0.05$) to that in splenocytes from mice

FIG. 3. Chimeric HBsAg DNA encoding a CTL epitope of HPV16 E7 elicits E7-directed CTL responses and protects against challenge with E7-expressing tumor. (a to c) A2.1K^D mice (three per group) were immunized twice i.d. with pRAH Δ IPQ DNA or with pHBsAgWT DNA or once s.c. with RAH peptide plus adjuvant. IFN- γ -secreting splenocytes harvested at 14 days (DNA immunizations) or 10 days (peptide immunizations) were quantified by ELISPOT assay with or without specific peptide (RAH) as shown. Bars are means $\pm$ standard errors of three replicates. (d to f) CTL responses following immunization with pRAH Δ IPQ DNA. Mice (three per group) were immunized as described above. Percent cytotoxicity of splenocytes restimulated with RAH peptide was measured in a ^{51}Cr release assay using EL4.A2 target cells pulsed with or without RAH peptide. Data points are means $\pm$ standard deviations of three replicates (although plotted, standard deviation bars are too small to appear). (g and h) Growth of E7-expressing tumor in immunized mice and controls. Mice immunized with pRAH Δ IPQ DNA, with pHBsAg wild-type DNA, or with RAH peptide plus adjuvant and unimmunized mice were challenged with 2×10^5 E7-expressing TC-1 tumor cells on day 14 postimmunization. Results are expressed as tumor-free mice (%) at the indicated time points. Groups of tumor-challenged mice were compared by use of the log rank statistic.

immunized with a single recombinant encoding M2₁₅ alone (pM2₁₅ Δ IPQ) (Fig. 4a). The RAH-directed IFN- γ response was lost ($P < 0.001$) in mice immunized with the double recombinant compared with that in mice immunized with the single recombinant encoding the RAH epitope alone (pRAH Δ GLS) (Fig. 4b).

Restimulated splenocytes from mice immunized with pM2₁₅ Δ IPQ.RAH Δ GLS.HW1 killed target cells pulsed with either M2₁₅ peptide or RAH peptide (Fig. 4c and d). While the restimulated splenocytes killed RAH-expressing target cells significantly more than target cells not expressing RAH (50:1 effector:target cell ratio; $P < 0.001$) (Fig. 4d), the killing was

less than that observed with restimulated splenocytes from mice immunized with pRAH Δ GLS encoding only one inserted RAH CTL epitope ($P < 0.001$). (Note that RAH-restimulated splenocytes from pHBsAgWT-immunized mice fail to kill RAH-expressing target cells [data not shown].)

In view of the down-regulated CTL response to the RAH epitope in mice immunized with pM2₁₅ Δ IPQ.RAH Δ GLS.HW1, we asked whether these mice were nonetheless protected against RAH-expressing tumor. Mice immunized with the double recombinant pM2₁₅ Δ IPQ.RAH Δ GLS.HW1 or with the single recombinant pRAH Δ GLS were fully protected against E7-expressing tumor. In contrast, 40 to 60% of unvaccinated mice or mice immunized with plasmids encoding wild-type HBsAg or chimeric HBsAg not containing the RAH epitope developed tumor within 8 days (Fig. 4c).

Together, these data indicate that immunization with rHBsAg DNA encoding two foreign CTL epitopes from different pathogens elicits effector and memory CTL responses to both epitopes. Although the RAH-directed CTL response measured in vitro was down-regulated in mice immunized with the chimeric HBsAg DNA encoding two epitopes, these mice were nonetheless protected against tumor challenge as effectively as mice immunized with the chimeric HBsAg DNA encoding the RAH epitope alone.

Epitope replacement allows for efficient formation and secretion of VLPs. DNA vaccines induce CTL responses through the classical endogenous pathway via intracellular translation and antigen processing of the proteins they encode. In addition, HBsAg DNA vaccines may induce CTL through the alternative exogenous pathway via secreted HBsAg VLPs (9, 28, 38, 39). It was thus relevant to ask if our strategy for deriving chimeric HBsAg DNA vaccines encoding foreign CTL epitopes also allowed for the formation of secreted HBsAg VLPs. Previous studies to derive HBsAg VLP immunogens containing foreign CTL epitopes have explored the insertion of foreign domains into the pre-S2 region (15). Indiscriminate insertions into HBsAg may lead to unpredictable stability and secretion, probably due to conformational changes in the recombinant HBsAg molecule (11). Thus, we reasoned that deletion of HBsAg-specific CTL epitopes and their replacement with foreign CTL epitopes with similar physical properties (i.e., size and hydrophobicity) would result in less structural change and thereby help to conserve HBsAg conformation and subsequent stability/secretability. To test this supposition, we therefore evaluated whether pM2₉ Δ IPQ, pM2₁₅ Δ IPQ, and pM2₁₅ Δ GLS encoded structurally authentic VLPs. We did this by evaluating the capacity of pM2₉ Δ IPQ, pM2₁₅ Δ IPQ, and pM2₁₅ Δ GLS to package HDag-L for secretion. HDV is an RNA agent which exists as a subviral satellite of HBV. Coexpression of HDag-L and HBsAg leads to incorporation (packaging) of HDag-L into the empty HBsAg-S VLPs and to secretion (3, 34, 46). We cotransfected DNAs encoding HDag-L and the chimeric HBsAg polypeptide into HuH-7 cells and subsequently determined the presence of secreted HDag-L in the cell culture supernatant by immunoblotting. HDag-L was confirmed in the supernatant of cells which had been cotransfected with any one of the chimeric HBsAg (Fig. 5a, lanes 1 to 3), although to a lesser extent than in cells cotransfected with wild-type HBsAg (Fig. 5a, lane 4). No HDag-L was

FIG. 4. Immunization with chimeric HBsAg DNA encoding two protective CTL epitopes from distinct pathogens elicits CTL responses to both epitopes. (a and b) Epitope-specific IFN- γ -secreting response of ex vivo splenocytes following two i.d. immunizations of mice (BALB/c $\times$ A2.1K^b; three per group) with chimeric HBsAg DNA encoding both M2₁₅ and RAH epitopes (pM2₁₅ΔIPQ.RAHΔGLS.HWI), the M2₁₅ epitope alone (pM2₁₅ΔIPQ), or the RAH epitope alone (pRAHΔGLS). IFN- γ -secreting cells were quantified by ELISPOT assay using splenocytes incubated for 15 to 18 h with or without specific peptide M2₁₅ or RAH as shown. Histogram bars represent means $\pm$ standard deviations of three replicates. (c and d) Percent cytotoxicity of splenocytes restimulated with RAH peptide or with M2₁₅ peptide measured in a ⁵¹Cr release assay using P815 or EL4.A2 target cells pulsed with M2₁₅ peptide or RAH peptide, respectively, or without peptide, as shown. Data points represent means $\pm$ standard deviations of three replicates (standard deviation bars, although plotted, are too small to appear in some cases). (e) Growth of E7-expressing tumor in mice immunized with pM2₁₅ΔIPQ.RAHΔGLS.HWI DNA, with pRAHΔ IPQ DNA, or with control DNA as indicated. Mice (five per group) were challenged with 2×10^5 E7-expressing TC-1 tumor cells on day 14 postimmunization. Results are expressed as tumor-free mice (%) at the indicated time points. Differences between means (*, **, ***) were calculated using Student's *t* test. Differences between survival curves (****) were calculated using the log rank statistic.

detected in the supernatant of cells transfected only with the plasmid encoding HDAG-L, although HDAG-L was clearly present in the cell pellet, confirming that in the absence of HBsAg, HDAG-L was not secreted (Fig. 5b, lane 6). This result indicates that chimeric HBsAg polypeptides in which the HBsAg-specific IPQ or GLS CTL epitopes are replaced by a foreign CTL epitope (M2) retain the capacity for assembly and secretion of structurally sound HBsAg particles.

DISCUSSION

A limited range of antigen delivery techniques are available to prime precursor CTLs to recombinant antigens. These include infection with recombinant replicating (bacterial or viral) vectors, the inoculation of antigen-encoding plasmid DNA or RNA, the association of protein antigen with selected adjuvants (e.g., squalene, Iscom), the injection of VLPs, and the

FIG. 5. Chimeric HBsAg containing foreign CTL epitopes package HDaAg-L for secretion. Culture fluid from HuH-7 cells cotransfected with plasmids encoding chimeric or wild-type HBsAg proteins, and a plasmid encoding HDaAg-L, was pelleted through sucrose cushions and resuspended in sample buffer, and supernatants (a) and cell pellets (b) were analyzed by immunoblotting using a human serum antibody specific for HDaAg-L. Lanes: 1, pM2₉ΔIPQ; 2, pM2₁₅ΔIPQ; 3, pM2₁₅ΔGLS; 4, wild-type pHBsAg; 5, wild-type pHBsAg without pHDaAg-L; 6, pHDaAg-L without pHBsAg; 7, neither pHDaAg-L nor pHBsAg.

injection of naked lipoprotein particles. HBsAg is the already licensed human vaccine for hepatitis B virus infection, making it an attractive vehicle for the delivery of epitopes from other pathogens and diseases, from both a regulatory and a scientific point of view. While HBsAg has been used experimentally as a carrier in recombinant vaccine studies, this primarily has been in the delivery of a variety of foreign B-cell epitopes for antibody induction, most commonly by the insertion of these epitopes into the external hydrophilic loop (5, 23, 29, 33, 45). There are few reports of exploiting the inherent powerful immunogenicity of HBsAg to deliver foreign CTL (2, 15, 25) or T-helper (5) epitopes, and this likely relates to difficulties arising in stability and antigen processing that are incurred by the insertion of foreign sequences into hydrophobic regions of HBsAg, of which at least some are essential for correct conformation (13).

Comparison of wild-type HBsAg delivered as a DNA immunogen and that delivered as a VLP immunogen suggests that DNA is the preferred modality both economically and practically and in terms of immunogenic efficacy, at least for CTL induction (6). That a single intramuscular injection of HBsAg plasmid DNA gives CTL detectable for many months postvaccination (7, 9) suggests that continuous exposure to small doses of antigen provided from ongoing DNA transcription may be necessary to maintain immunity. Injected DNA encoding HBsAg DNA is likely to transfect a variety of cells, though only the transfection of professional antigen-presenting cells (APCs) leads to activation of the CTL response via the intracellular (endogenous) processing pathway (38). Particle formation within the APC is not an essential requirement for this to occur (38). Secretion of HBsAg particles (e.g., by DNA-

transfected muscle cells) allows uptake of secreted particles by APCs for processing via the exogenous pathway (8), thus providing a second mechanism for HBsAg CTL generation (41). After a single intramuscular injection of HBsAg DNA, the antigen can be found in circulation for at least 1 month, suggesting sustained expression in muscle fibers (26). HBsAg is likely to persist elsewhere, e.g., in follicular dendritic cells, accounting for the sustenance of a prolonged immune response (17). The powerful CTL responses induced by HBsAg DNA vaccination may be explained by prolonged and higher expression, secretion for uptake of particles by APC, superior antigen processing, and the presence of multiple T-helper epitopes. Thus, DNA-based HBsAg immunization is extremely potent and may have an immune potential the same as that of natural infection (39). HBsAg-based DNA vaccination requires a dose as much as 2,500-fold lower than that used in previous clinical trials with conventional DNA (37). The fact that long-lasting CTL and antibody are efficiently induced in neonatal mice with wild-type HBsAg-encoding plasmid DNA (but not HBsAg particles) (41) argues in favor of a DNA vaccine approach for recombinant HBsAg vaccines.

In the present study, we sought to exploit the inherent immunogenicity of HBsAg DNA as a carrier for the delivery of disease-relevant foreign CTL epitopes. We argued that the simplistic approach of inserting CTL epitope-encoding sequences into the HBsAg gene at sites where restriction endonuclease sites fortuitously exist, not accounting for spatial requirements and the tertiary configuration of the HBsAg polypeptide, is likely to yield unpredictable outcomes, and this has hitherto constrained the exploitation of HBsAg for the delivery of inserted foreign CTL epitopes. We reasoned that by deleting endogenous CTL epitopes and replacing them with foreign CTL epitopes of similar size and physical characteristics (hydrophobicity), we might avoid deleterious effects on the structural integrity of the recombinant HBsAg protein. Furthermore, we reasoned that targeting foreign CTL epitopes to sites of proven HBsAg CTL epitopes might predispose to appropriate antigen processing of insert.

A number of HBsAg CTL epitopes in hydrophobic regions of the HBsAg molecule, restricted through murine (39, 42) and human (28) MHC-I molecules, have been described previously. We elected to delete two of the stronger of these endogenous CTL epitopes (IPQ and GLS [Table 2]) and replace them with protective foreign epitopes from the M2 protein of RSV and/or the E7 protein of HPV16.

We show that replacement of DNA encoding the endogenous IPQ epitope with DNA encoding either RSV M2₉ or M2₁₅ epitopes or the HPV oncoprotein RAH epitopes produced chimeric HBsAg DNA immunogens which elicited effector and memory CTL responses to the foreign CTL epitope (Fig. 1, 3, and 4). Moderate effector CTL responses measured by IFN- γ release (Fig. 1a and b) translated into strong memory responses when specific antigen was reencountered (Fig. 1f and g), as has been seen in other systems (47). We show that M2-specific CTL responses were associated with reduced proliferation of virus in the lungs of RSV-challenged mice and with amelioration in pulmonary pathology. It has been previously demonstrated that protection against RSV afforded by an RSV M2 CTL epitope vaccine is associated with CTLs which kill RSV-expressing cells in vitro (22). Similarly, RAH-specific

CTL responses were associated with protection against challenge with an E7-expressing tumor. We further show HBsAg recombinant for both RSV and HPV CTL epitopes elicited simultaneous responses to both epitopes. However, there was evidence of immunodominance of the RSV M2 epitope. (Interestingly, immunodominance of the endogenous HBsAg-specific epitope at the site of the M2 insertion has also been reported [42].) While the memory CTL response to RAH was down-regulated in mice immunized with HBsAg encoding the M2 and RAH epitopes, the mice were still protected against tumor challenge (Fig. 4e). The phenomenon of relatively small CTL responses, as measured by *in vitro* cytotoxicity assay, translating into substantial tumor protection *in vivo* has been observed for other tumor vaccination systems in our laboratory (unpublished observations). Nonetheless, we have preliminary data to indicate that immunization with chimeric HBsAg DNA encoding three foreign epitopes elicits CTL responses to all three (not shown). Together, these data suggest the applicability of HBsAg as a vector for the delivery of protective CTL responses to human disease-relevant foreign epitopes and also suggest the possibility of simultaneous protection against multiple diseases with a single polyvalent HBsAg vaccine. The approach of delivering a limited number of MHC-I-restricted epitopes to target multiple diseases in humans by use of HBsAg DNA as a vector is feasible, since (i) at least 15 HBsAg CTL epitopes are candidates for replacement (28, 32, 48); (ii) in general, CTL-mediated pathogen and tumor protection is remarkably parsimonious in terms of the number of CTL epitopes to which the responses must be directed (19, 36); and (iii) a limited number of major MHC-I supertypes account for the majority of MHC polymorphism, allowing broad population coverage with very few disease epitopes (43). Furthermore, immunization with mixtures of chimeric HBsAg DNAs encoding different disease-relevant epitopes may be employed to enhance epitope delivery capacity.

CTL responses to foreign epitopes delivered in the context of chimeric HBsAg DNA compared favorably to those induced by immunization with a vast molar excess of epitope peptide plus adjuvant. This latter approach induces maximal (though short-lived) CTL responses and is a standard against which other CTL-inducing modalities may be compared. In view of the superior immunogenicity of HBsAg-based vaccines (see above) compared to that of conventional vaccine approaches, it would be interesting to compare the numbers of cognate CD8⁺ CTLs in the lungs of recombinant HBsAg-vaccinated mice and peptide-plus-adjuvant-vaccinated mice following RSV challenge. Additionally, we observed that responses to epitopes inserted into HBsAg could exceed the observed response to the deleted HBsAg epitope (following wild-type HBsAg immunization) which they replaced. These observations suggest that the choice of sites for inserting replacement foreign epitopes within the HBsAg molecule need not be overly influenced by the magnitude of the response to the endogenous HBsAg epitope situated at that site. Response is likely to be a function not only of primary amino acid sequence but also of the context of the epitope within the protein, such that intramolecular competition for cellular processing and HLA binding influences the magnitude of the response (35). It is also noteworthy that an HBsAg CTL epitope can be replaced with a foreign CTL epitope restricted through a different

MHC-I haplotype to elicit strong insert-directed CTL responses.

In spite of our approach of replacing HBsAg-specific CTL epitopes with the M2 CTL epitope, the assembly and/or stability and secretion of recombinant HBsAg was reduced compared with those of wild-type particles (measured as reduced capacity to package hepatitis delta virus large antigen for secretion [Fig. 5]). The inference is that the replacement insertions interfere with the assembly or stability of structurally appropriate HBsAg particles. Length of insert is the major factor determining the level of secretion (11). However, it is noteworthy in our study that impaired secretion of functionally intact HBsAg particles occurred even when the replacement of the HBsAg-specific CTL epitope with foreign epitope resulted in no net change in the number of amino acid residues in the chimeric HBsAg compared to that in the wild type (pM2₉ΔIPQ [Fig. 5]). This underscores the importance of specific sequence in topogenic regions essential for particle assembly (11, 13). In preliminary experiments (not shown), we found that antibody induction to a B mimotope of RSV (Table 1) inserted into the hydrophilic a determinant was lost when CTL epitopes were inserted at the IPQ and GLS sites (pM2₁₅ΔIPQ.RAHΔGLS.HW1) (Table 2).

Immunization with plasmid HBsAg DNA evokes cytokines which predispose to a Th1-type response (26). Provision of T help for primary effector responses may be provided by HBsAg-specific T-helper epitopes (2, 30) or CpG dinucleotides in bacterial DNA (10). The recall of CTL-epitope specific memory (required when the pathogen or disease against which the chimeric HBsAg vaccine is directed is encountered) is likely to be T help independent (reviewed in reference 47).

In summary, using a strategy of deletion of DNA encoding HBsAg-specific CTL epitopes and replacement with DNA encoding foreign CTL epitopes, we have derived chimeric HBsAg DNA immunogens which elicited foreign epitope-directed effector and memory CTL responses associated with pathogen- and tumor-protective responses *in vivo*. We show that HBsAg recombinant for more than one CTL epitope elicited simultaneous responses to both epitopes. There are generic implications for the application of HBsAg, which is already licensed as human vaccine, as a vector for the delivery of multiple disease-relevant protective CTL responses. There are also specific implications for HBsAg-vectored vaccines for RSV- and HPV-associated tumors.

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W.-P. Woo and T. Doan contributed equally to the experimentation and should be regarded as joint first authors.

H.-J. Netter and R. W. Tindle contributed equally to the design and implementation of the study and should be regarded as joint senior authors.

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