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Nucleic Acid Vaccination Primes Hepatitis B Virus Surface Antigen-Specific Cytotoxic T Lymphocytes in Nonresponder Mice

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The efficiency of different vaccination techniques to prime in vivo major histocompatibility complex class I-restricted murine cytotoxic T-lymphocyte (CTL) precursors to hepatitis B virus small surface antigen (HBsAg) was investigated. Mice were immunized either by injection of a low dose of recombinant HBsAg protein preparations (native HBsAg particles or denatured HBsAg monomers) without adjuvants, by infection with recombinant vaccinia virus carrying an HBsAg-encoding gene, or by intramuscular transfer of plasmid DNA encoding HBsAg under appropriate promoter control. In *H-2*^{d} mice, an L^d-restricted, S$_{28-39}$-specific CTL response was efficiently primed by all alternative vaccination techniques tested, but the most potent priming of class I-restricted CTL to HBsAg in vivo was observed with DNA immunization. Priming of anti-HBsAg CTL in *H-2*^{b} mice was not detectable after infection with a recombinant vaccinia virus or after injection with exogenous recombinant HBsAg preparations. After DNA immunization, however, both K^b- and D^b-restricted CTL reactivity to HBsAg emerged in *H-2*^{b} mice. Hence, nucleic acid immunization revealed class I-restricted CTL responsiveness to HBsAg in a mouse strain previously considered to be a nonresponder at the CTL level. These results demonstrate that the simple technique of nucleic acid immunization not only is extremely efficient but also reveals an extended spectrum of potentially immunogenic epitopes of protein antigens.

Nucleic acid immunization is an efficient technique to define immunogenic epitopes of proteins specifically recognized by B or T cells. It involves the injection into the animal of DNA containing protein-encoding sequences cloned under appropriate promoter control. These constructs are injected into tissues that support the in vivo expression of the plasmid-encoded protein and its immunogenic presentation. Protein antigen-encoding plasmids expressed in skin (19, 25), muscle (4–6, 11, 13, 27–29), and endothelium and vascular smooth muscle (15–18) have been shown to prime immune responses. The successful induction of major histocompatibility class I-restricted responses of cytotoxic T lymphocytes (CTL) by DNA immunization has been reported (16, 24, 27, 30).

We have described the efficient priming of murine class I (L^d)-restricted CTL by infection with a recombinant vaccinia virus that expresses the hepatitis B virus (HBV) surface antigen (HBsAg) (14) and by the injection of a low dose of native HBsAg particles (20, 21) or denatured HBsAg monomers (22). Immunization with even high concentrations of a synthetic peptide corresponding to the antigenic S$_{28-39}$ epitope did not prime CTL in vivo (22). The induction of CTL by soluble HBsAg particles and HBsAg monomers was unexpected because exogenous protein antigens are generally thought to be excluded from the endogenous processing pathway (reviewed in references 7, 8, 26, and 31) in which peptides are generated for class I-restricted presentation. In the endogenous processing pathway, peptides are released during cytosolic degradation. In studying processing of HBsAg particles for class I-restricted epitope presentation to T cells, we defined a novel endosomal pathway of processing of exogenous HBsAg particles for class I-restricted epitope presentation (23).

In this study, we compared the relative efficiencies of in vivo priming of class I-restricted murine CTL to HBsAg by using different vaccination strategies. HBsAg was delivered to mice either by injecting a low dose of native HBsAg particles or denatured HBsAg monomers (without adjuvants), by infecting mice with a recombinant vaccinia virus carrying an HBsAg-encoding gene, or by injecting plasmid DNA that expresses the HBsAg gene under appropriate promoter control. We tested whether class I-restricted presentation of epitopes derived from HBsAg for CTL priming in vivo is more efficient in one of the alternative vaccination techniques tested. We also examined whether the definition of responder versus nonresponder status defined for class I-restricted CTL reactivity to HBsAg in different strains of inbred mice depends on the vaccination strategy used. We found that DNA immunization is the most efficient way to prime class I-restricted CTL to HBsAg in vivo and that this technique reveals class I-restricted CTL responsiveness in mouse strains previously considered to be nonresponsive to HBsAg at the CTL level. *H-2*^{b} strain mice do not show evidence of priming of anti-HBsAg CTL after infection with recombinant vaccinia virus or after injection with exogenous recombinant HBsAg preparations. In contrast, these animals show *K*^{b}- as well as *D*^{b}-restricted CTL reactivity to HBsAg after DNA immunization. Hence, nonresponsiveness of class I-restricted CTL is relative and can be overcome by an appropriate choice of antigen delivery.

MATERIALS AND METHODS

Mice. BALB/cJ (*H-2*^{d}), B10.D2 (*H-2*^{d}), and C57BL/6 J (*H-2*^{b}) mice were bred and kept under standard-pathogen-free conditions in the animal colonies of Ulm University (Ulm, Germany) and The Scripps Research Institute (La Jolla, Calif.).

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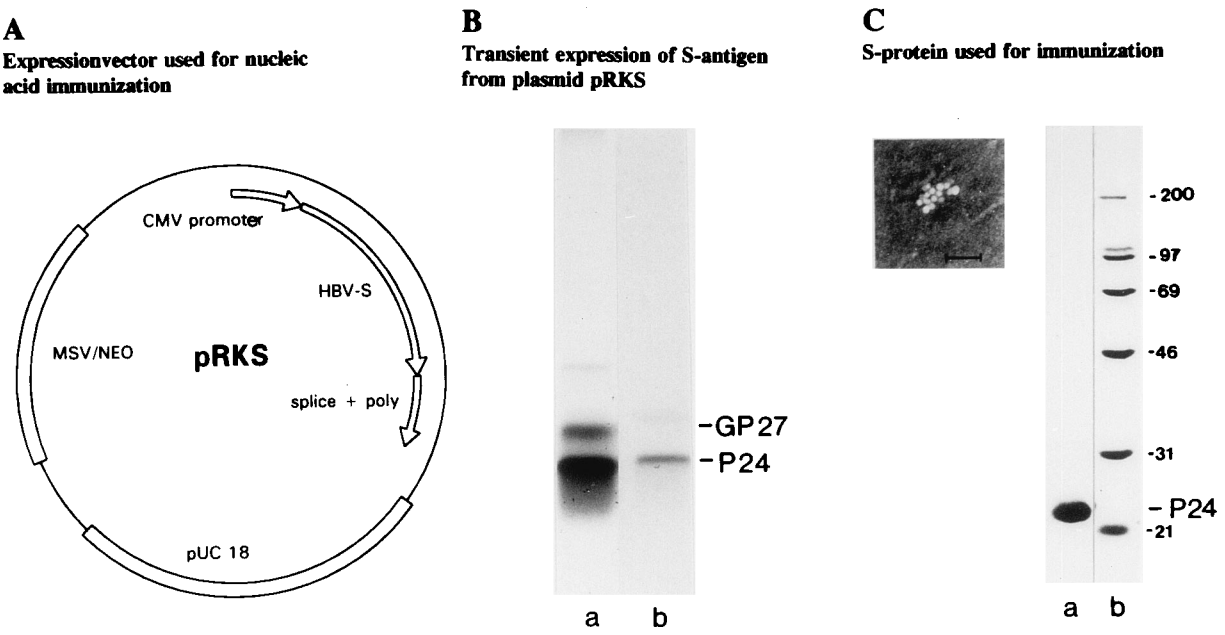

FIG. 1. Nucleic acid immunization and protein immunization. (A) The small S antigen of HBV was cloned into the expression vector pRK as described in Materials and Methods. CMV, cytomegalovirus; MSV/NEO, murine sarcoma virus *neo* gene. (B) Expression of the HBsAg from plasmid pRKS was tested in transiently transfected murine fibroblasts. Cells were transfected with plasmid pRKS, using DOTAP-liposomes (Boehringer, Mannheim, Germany). After 48 h, cells were labeled with [^{35}S]methionine, and HBsAg particles were precipitated from cell lysates (lane a) or from growth medium (lane b) by using a polyclonal rabbit anti-S antibody and protein A-Sepharose. Under reducing SDS-PAGE conditions, the nonglycosylated p24 and the glycosylated gp27 gene products were detected in cell lysates as well as in the supernatant. (C) *Hansenula*-derived HBsAg particles were characterized by electron microscopy (bar, 100 nm). Particulate HBsAg was disrupted with SDS and mercaptoethanol, and HBsAg monomers (p24) were gel purified as described previously (21). The gel-purified HBsAg monomers were analyzed by SDS-PAGE and Western blotting with a polyclonal rabbit anti-S serum and ^{35}S-labeled protein A (lane a). ^{14}C-labeled molecular weight markers (lane b) ranged in size from 200 to 21 kDa, as indicated on the right.

Breeding pairs of these mice were obtained from Bomholtgard (Ry, Denmark) and Jackson Laboratory (Bar Harbor, Maine). Male and female mice were used at 12 to 16 weeks of age.

Vaccinia virus. Vaccinia-HBs virus, a recombinant vaccinia virus (3) encoding the HBsAg sequence (subtype ayw), was a generous gift of H. J. Schlicht (Ulm, Germany). The ability of this virus to induce HBsAg-specific CTL in immunized B10.D2 mice has been previously described (1–3).

Recombinant S antigen. The methylotrophic *Hansenula polymorpha* yeast system was used for the production of recombinant HBsAg. The HBsAg (subtype adw 2) S sequence was inserted into the expression vector pMPT121 and transformed into *H. polymorpha* host strain RB10 (10). Expression of the HBsAg-encoding gene under control of a methanol-inducible promoter yielded high intracellular levels of nonglycosylated HBsAg (5 to 8% of the total cell protein). Yeast transformants efficiently expressing HBsAg were established as strains for fermentation. Yeast cells were disrupted in a large-scale commercial bead mill, and the crude extract was cleared by centrifugation. HBsAg S protein self-assembled into 22-nm subviral particles after the disruption of yeast cells. HBsAg particles were purified by adsorption to silica gel, column chromatography, and isopycnic ultracentrifugation. HBsAg particle preparations were characterized by electron microscopy and by Western blotting (immunoblotting) as described previously (20–22). Recombinant HBsAg S protein produced in the *Hansenula* yeast system contained exclusively the nonglycosylated p24 S protein assembled into 22-nm particles (10). Treatment of S particles with detergents under reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) conditions completely disrupted them into p24 monomers (22, 23) (Fig. 1C). Gel-purified p24 S monomers were used in immunization experiments using denatured HBsAg monomers.

Construction of the HBsAg-encoding expression vector pRKS used for nucleic acid immunization. The small-S-antigen-encoding 935-bp *Xho*I-*Nsp*I fragment (nucleotides 3053 to 2118; the unique *Eco*RI site was set at position 3182) of HBV (subtype ayw) from plasmid pTKTHBV2 (a generous gift of M. Meyer, Munich, Germany) was cloned into the multiple cloning site of plasmid pRK, creating pRKS (Fig. 1A). In this construct, the HBsAg-encoding gene is expressed under cytomegalovirus promoter control. Plasmid pRKS further contained the neomycin resistance (*neo*) gene. Plasmid DNA used for immunization was purified by anion-exchange chromatography, using a Qiagen maxiprep kit (Diagen, Hilden, Germany).

Cell lines. The *H-2*^{d} mastocytoma cell line P815 (TIB64) was obtained from the American Tissue Culture Collection (Rockville, Md.). The human (TAP)-deficient cell line T2 and its transfected subclones T2.L^d, T2.K^b, and T2.D^b were a generous gift from P. Cresswell (New Haven, Conn.). The *H-2*^{b} T-lymphoma cell line RBL5 was provided from H.-U. Weltzien (Freiburg, Germany). The *H-2*^{b} EL4 cell line was obtained from the American Type Culture Collection. This cell line was transfected with an Epstein-Barr virus-based vector that expresses the HBV (subtype ayw) large envelope polypeptide as previously described (9). These transfected cells (EL4-HBVenv cells) were maintained under continuous selective pressure except when cocultivated with primed spleen cells. P815 cells that express the HBV (subtype ayw) large envelope polypeptide under the control of the mouse mammary tumor virus-Rous sarcoma virus promoter complex (P815-HBVenv cells) were similarly maintained as previously described (2, 3). The bovine papillomavirus-based vector BMGNeo (a generous gift from Y. Karasuyama and F. Melchers, Basel, Switzerland) was treated with the restriction enzymes *Xho*I and *Bam*HI, deleting the poly(A) signal of rabbit β-globin (12). The *Xho*I-*Bgl*II fragment of HBV (subtype ayw) from plasmid TKTHBV2, encoding the small-S-antigen sequence up to the poly(A) signal, was cloned into the BMGNeo vector, creating the BMG/HBS vector. BMG/HBS vector DNA or nonrecombinant BMGNeo vector DNA was transfected into murine cell lines as described in detail elsewhere (20). Stable expression of the HBV small surface antigen was demonstrated in cloned and subcloned P815/S and RBL5/S cells (20).

HBsAg particle and HBsAg monomer immunization. Mice were immunized intraperitoneally or subcutaneously into the base of the tail with the recombinant native HBsAg particles or denatured, gel-purified HBsAg monomers (1 to 50 μg per mouse). Spleen cells (from intraperitoneally immunized mice) or lymph node cells (from subcutaneously immunized mice) were obtained 3 weeks postimmunization.

Infection of mice with recombinant vaccinia-HBs virus. Mice were immunized twice with 5×10^7 PFU of recombinant vaccinia-HBs virus intraperitoneally at 7-day intervals. Spleens were harvested 1 week after the second immunization.

Nucleic acid immunization. We injected 50 μl of phosphate-buffered saline (PBS) containing 50 μg of pRKS plasmid DNA into each regenerating tibialis anterior muscle 5 days after the injection of cardiotoxin (Latoxan, Rosans, France) as previously described (4, 5). All mice received bilateral intramuscular injections once. Spleen cells were obtained from immunized mice 3 weeks postimmunization.

In vitro pulse of antigen-presenting cells with HBsAg particles. In vitro sensitization of antigen-presenting cells was carried out in PBS supplemented with 0.3% bovine serum albumin as described in detail elsewhere (23). Cells were incubated with recombinant HBsAg particles (1 to 10 μg of S protein per 10^6 to

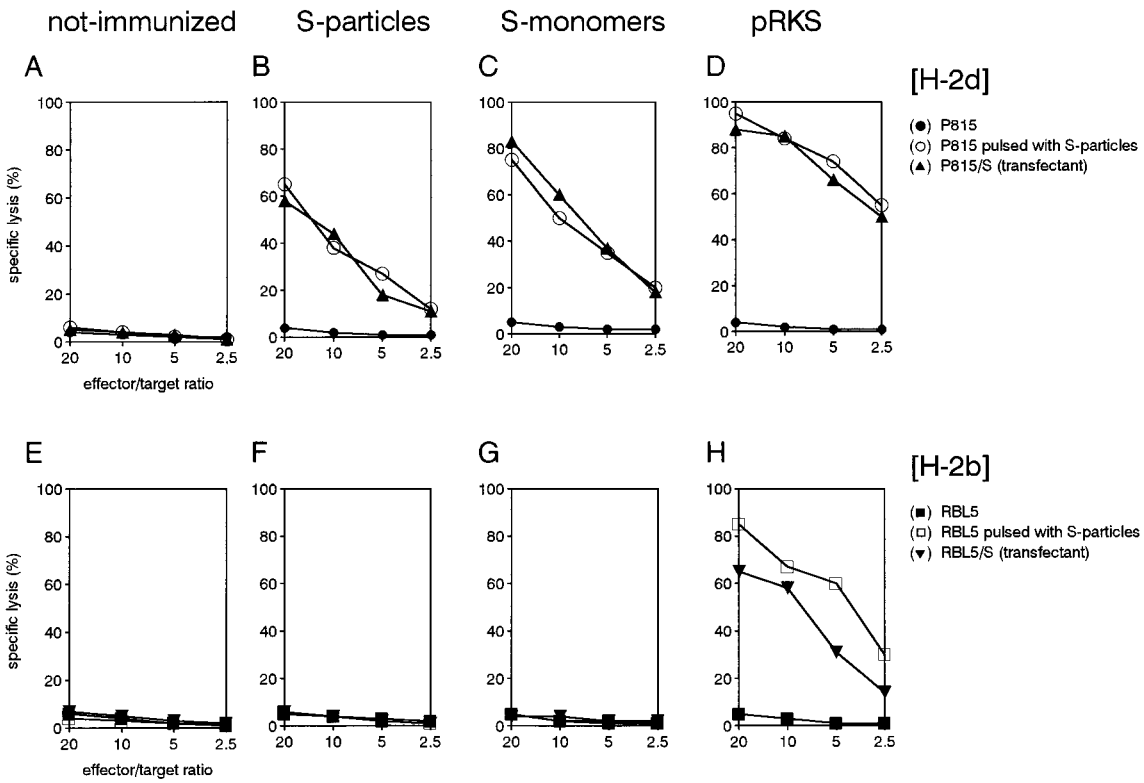

FIG. 2. The class I-restricted T-cell response of *H-2*^{d} and *H-2*^{b} mice to HBsAg. *H-2*^{d} BALB/c (A to D) or *H-2*^{b} C57BL/6 (E to H) mice were not immunized (A and E), injected subcutaneously with 1 μg of recombinant, native HBsAg S particles without adjuvants (B and F), injected subcutaneously with 1 μg of recombinant, denatured HBsAg S monomers (C and G), or injected with 2 × 50 μg of plasmid DNA encoding HBsAg (D and H). Three weeks postimmunization, lymphoid cells were prepared from these mice (from spleen or lymph nodes) and restimulated in vitro with syngeneic, HBsAg particle-pulsed P815 (*H-2*^{d}) (A to D) or EL4 (*H-2*^{d}) (E to H) cells. After a 5-day in vitro culture, effector cells were harvested and tested in a conventional cytotoxicity assay against HBsAg particle-pulsed P815 (A to D) or RBL5 (E to H) cells or HBsAg-expressing P815/S (A to D) or RBL5/S (E to H) transfectants. The lysis values shown at the indicated effector/target ratios represent means of triplicates.

10^7 cells per 500 μl) for 1 h at 37°C. Subsequently, cells were extensively washed and used as stimulator or target cells (23).

Cytotoxicity assay. Soluble HBsAg- and DNA-primed spleen cells or lymph node cells were suspended in alpha minimal essential medium tissue culture medium supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 10% (vol/vol) fetal calf serum (Pan Systems, Aidenbach, Germany). Responder cells (3×10^7) were cocultured with 1.5×10^6 syngeneic, HBsAg-expressing transfectants (20) or HBsAg particle-pulsed cells (irradiated with 20,000 rad) in 10 ml of medium in upright 25-cm^2 tissue culture flasks in a humidified atmosphere containing 7% CO_2 at 37°C. Vaccinia-HBs virus-primed cells were harvested 7 days after the last injection, and splenocytes (4×10^6 per well) were cultured with irradiated (20,000 rad) P815-HBVenv transfectants (10^5 per well) in complete EHAA culture medium (GIBCO, Frederick, Md.) containing antibiotics, 2-mercaptoethanol, 10% fetal calf serum, and 2.5% EL4 supernatant, as a source of T-cell growth factor, in 24-well plates (Corning Glass Works, Corning, N.Y.) as previously described (2, 3). Cytotoxic effector populations were harvested after various intervals of in vitro culture and washed twice. Serial dilutions of effector cells were cultured with 2×10^3 ^{51}Cr-labeled targets in 200-μl round-bottom wells. Specific cytolytic activity of cells was tested in short-term ^{51}Cr release assays against particle-pulsed or transfected targets and compared with the activity of nonpulsed and nontransfected controls. After a 4-h incubation at 37°C, 100 μl of supernatant was collected for γ-radiation counting. The percent 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 less than 15% of the total counts. Data shown are the means of triplicate cultures. The standard error of the mean of triplicate data was always less than 15% of the mean.

Determination of serum antibody levels. Antibodies against HBsAg were detected in mouse sera by using the commercially available test IMxAUSAB (Abbott, Wiesbaden, Germany). Antibody levels were quantified by using six standard sera (0 to 1,000 mIU/ml). The tested sera were diluted so that the measured optical density values were between those of standard sera 1 and 6. Values presented in this report were calculated by multiplying the serum dilution by the measured antibody level (milli-internation units per milliliter) (21, 22).

RESULTS

***H-2* haplotype-associated responsiveness of class I-restricted T cells to HBsAg defined by S-protein and recombinant vaccinia-HBs virus immunization.** BALB/c (*H-2*^{d}), B10.D2 (*H-2*^{d}), or C57BL/6 (*H-2*^{b}) mice were immunized with 1 μg of recombinant HBsAg particles or 1 μg of denatured HBsAg monomers without adjuvants or were infected with a recombinant vaccinia-HBs virus that expresses HBsAg and secretes HBsAg particles. These modes of immunization have all been shown to prime class I-restricted CTL specific for an epitope of HBsAg in *H-2*^{d} mice (2, 3, 14, 20, 22). Class I-restricted, HBsAg-specific cytolytic reactivity was detected in mice primed by both soluble HBsAg preparations after specific restimulation in vitro with syngeneic, HBsAg particle-pulsed or HBsAg-transfected cells. In vivo-primed lymph node cells from *H-2*^{d} mice were efficiently restimulated in vitro and efficiently lysed syngeneic cells either expressing a transfected HBsAg-encoding gene or pulsed with HBsAg particles (Fig. 2B, 2C and 3A). Class I-restricted, HBsAg-specific cytolytic activity was also detected in spleen cells from vaccinia-HBs virus-primed mice after 1 week of in vitro restimulation (38.9 and 1.4% specific lysis on B10.D2 and C57BL/6 mice, respectively). As previously described, the cytolytic response induced by immunization of *H-2*^{d} mice with both exogenous HBsAg preparations or by infection with vaccinia-HBs virus was mediated by $CD8^+$ CTL, restricted by L^d, and specific for the $S_{28\text{-}39}$ epitope (2, 14, 20, 22). The data shown in Fig. 2 demonstrate that

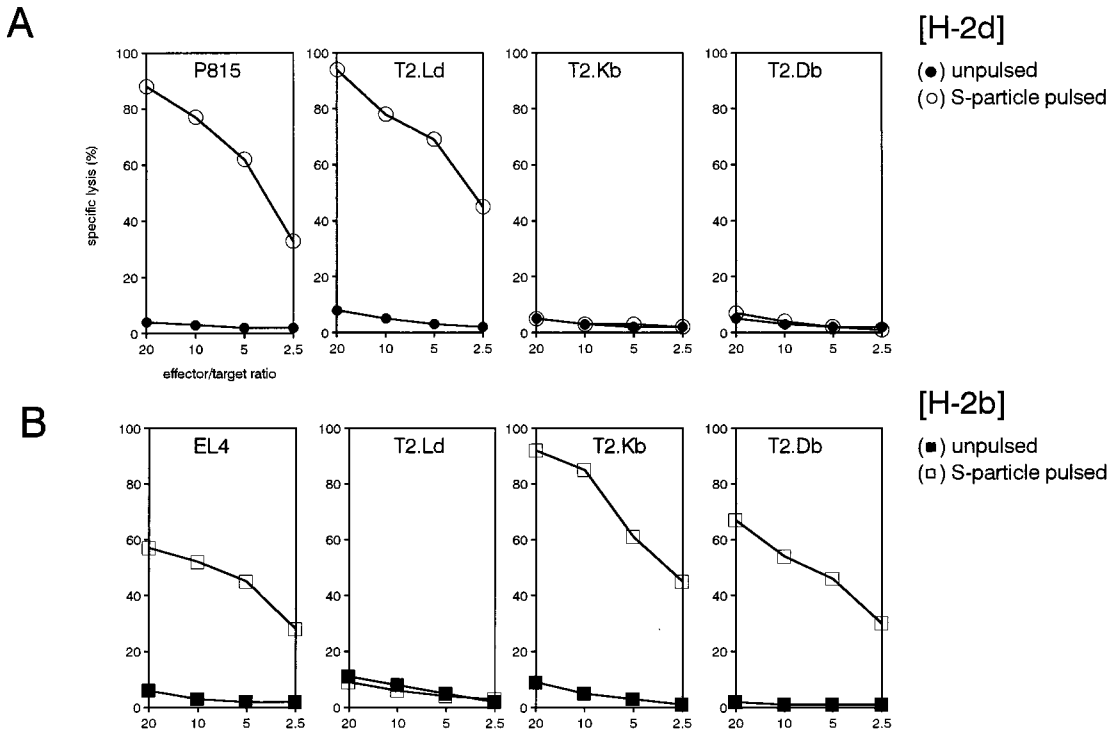

FIG. 3. Nucleic acid immunization primes K^b- and D^b-restricted CTL in *H-2*^{b} mice. The restriction specificities of CTL lines from *H-2*^{d} BALB/c (A) and *H-2*^{b} C57BL/6 (B) mice primed in vivo by nucleic acid immunization and restimulated in vitro with syngeneic, HBsAg particle-pulsed stimulator cells (*H-2*^{d} P815, [A] and *H-2*^{b} RBL5 [B]) were mapped. Human T.2L^d, T.2K^b, and T.2D^b cells, *H-2*^{d} P815 cells, and *H-2*^{b} EL4 cells were pulsed with HBsAg particles, washed, and used as targets. The plotted lysis values at the indicated effector/target ratios represent means of triplicates.

different antigen-presenting cells efficiently presented the class I-restricted epitope to CTL derived either from endogenously synthesized HBsAg or from exogenously supplied HBsAg particles (23).

Immunization of *H-2*^{b} (C57BL/6) mice with native HBsAg particles did not prime a CTL response in vivo (Fig. 2F), confirming our previously reported data (20). Furthermore, injection of denatured HBsAg monomers into *H-2*^{b} mice did not stimulate a CTL response (Fig. 2G). No evidence for CTL priming was detected in *H-2*^{b} mice when a higher dose of HBsAg (50 μg per mouse) was injected or when the HBsAg was injected intravenously or intraperitoneally (data not shown). Similarly, *H-2*^{b} mice immunized with the recombinant vaccinia-HBs virus failed to produce HBsAg-specific CTL even after 2 weeks of in vitro restimulation (80.2 and 1.9% specific lysis for B10.D2 and C57BL/6 mice, respectively; results reflect the average net percent specific lysis of ^{51}Cr-labeled stimulator cells after subtraction of the percent lysis of their nontransfected counterparts at an effector/target ratio of 10:1 [B10.D2] or 20:1 [C57BL/6]). Lymph node cells from unprimed *H-2*^{d} or *H-2*^{b} mice did not generate a primary in vitro cytotoxic response to HBsAg particle-pulsed or HBsAg-transfected targets after specific stimulation by coculture with the syngeneic, HBsAg particle-pulsed or HBsAg-transfected stimulator cells (Fig. 2A and E and data not shown). These data confirm and extend our observation that *H-2*^{d} mice develop, and *H-2*^{b} mice do not develop, specific class I-restricted CTL reactivity in response to immunization with different exogenous or virally expressed HBsAg preparations.

Induction of a class I-restricted CTL response to HBsAg by nucleic acid immunization. Mice were immunized by an injection of 2 × 50 μg of the HBsAg-encoding expression plasmid pRKS (Fig. 1A) into regenerating muscle. *H-2*^{d} mice generated a potent class I-restricted CTL response to this vaccination (Fig. 2D) that was identical in restriction and epitope specificity to the CTL response stimulated by HBsAg protein injection (Table 1). In BALB/c^{dm2} mice, which lack expression of L^d, no HBsAg-specific CD8$^+$ CTL were generated following immunization with HBsAg particles (20) or HBsAg-encoding plasmid DNA (data not shown). Confirming this finding, specific

TABLE 1. Epitope and restriction specificities of *H-2*^{d} CTL primed to HBsAg by nucleic acid immunization

Specificity	Antibody present in the cytotoxic assay[a]	% Specific lysis[b]		Pulsed[c]	Nonpulsed
		P815/S	P815		
Restriction		78	9		
	Anti-L^d	21	11		
	Anti-D^d	63	5		
	Anti-K^d	81	8		
Epitope	S_{28-39} 12-mer peptide			67	6

[a] The anti-L^d MAb 28-14-8S (HB27), the anti-D^d MAb 32-2-12S (HB 87), or the anti-K^d MAb 31-3-4S (HB77) was present in the culture medium during the cytotoxic assay at a concentration of 10 μg/ml.

[b] Spleen cells were obtained from *H-2*^{d} BALB/c mice immunized intramuscularly with plasmid pRKS DNA 3 weeks previously. Cells were restimulated in vitro and tested in the cytotoxicity assay against different targets. The cytotoxic response against *H-2*^{d} P815 cells, either expressing a transfected, HBsAg-encoding gene (P815/S) or pulsed with the antigenic S_{28-39} 12-mer peptide, was determined. Lysis values measured at an effector/target ratio of 20 are shown.

[c] P815 targets were either nonpulsed or pulsed for 60 min with the antigenic S_{28-39} 12-mer peptide of HBsAg (at a concentration of 10^{-8} M).

TABLE 2. Anti-HBsAg-specific cytotoxic effector cells primed by nucleic acid immunization in *H-2*^{b} mice display the CD4$^-$ CD8$^+$ phenotype

Treatment[a]	% Specific lysis[b]	
	HBsAg-pulsed EL4 cells	Nontreated EL4 cells
None	53	7
Complement	62	3
Anti-CD4 MAb + complement	48	10
Anti-CD8 MAb + complement	13	8

[a] Spleen cells were obtained from C57BL/6 mice immunized intramuscularly with pRKS plasmid DNA 3 weeks previously. These cells were either nontreated or treated with low-toxicity rabbit complement (catalog no. CL3051; Cedarlane, Hornby, Ontario, Canada), anti-CD4 MAb plus complement, or anti-CD8 MAb plus complement, as indicated. Cells were washed and cocultured for 5 days with inactivated RBL5 cells pulsed in vitro with HBsAg particles.

[b] Measured at an effector/target ratio of 20.

cytolytic reactivity of HBsAg-specific CD8$^+$ CTL generated by protein or DNA immunization was blocked by an anti-L^d monoclonal antibody (MAb) but not by an anti-K^d or anti-D^d MAb (Table 1) (20). All clones and subclones derived from these bulk lines were specific for the S_{28-39} epitope (Table 1) (23). Hence, the L^d-restricted S_{28-39} epitope is the only CTL epitope of HBsAg detectable in *H-2*^{d} BALB/c mice.

In contrast to the results obtained with HBsAg protein immunizations and with recombinant vaccinia-HBs virus infection, nucleic acid immunization primed a CTL response specific for HBsAg in *H-2*^{b} mice (Fig. 2H). The effector cells of this response were eliminated by anti-CD8 MAb-plus-complement treatment but not by anti-CD4 MAb-plus-complement treatment (Table 2); hence, they expressed the CD3$^+$ CD8$^+$ phenotype. HBsAg particle-pulsed *H-2*^{b} cells (RBL5 cells [Fig. 2H] or EL4 cells [Fig. 3B]) or HBsAg-transfected *H-2*^{b} RBL5/S cells (Fig. 2H) presented the epitope to *H-2*^{b}-derived CTL.

K^b- and D^b-restricted T-cell reactivities specific for HBsAg are revealed in *H-2*^{b} mice by nucleic acid vaccination. HBsAg particle-pulsed cells efficiently presented the L^d-restricted epitope to class I-restricted CTL from *H-2*^{d} mice. We have previously shown that exogenous HBsAg particles are processed in an novel endosomal/lysosomal pathway for L^d-restricted epitope presentation to CTL (23). This processing for L^d-restricted presentation of epitopes derived from exogenous HBsAg particles did not require the peptide transporter proteins TAP1 and TAP2 because it proceeded efficiently in TAP-defective T2.L^d cells (Fig. 3A) (23). T2 cells that expressed the wrong class I restriction element (T2.K^b or T2D^b cells) were not recognized by L^d-restricted CTL lines (Fig. 3A). Processing of exogenous HBsAg particles in this novel endosomal presentation pathway led to loading of antigenic peptides not only to L^d molecules but also to K^b and D^b molecules. HBsAg particle-pulsed T2.K^b or T2D^b cells efficiently presented epitopes to CTL from *H-2*^{b} mice primed by HBsAg-encoding plasmid DNA. Hence, immunization of *H-2*^{b} mice with HBsAg-encoding plasmid DNA primed HBsAg-specific CTL restricted by either K^b or D^b (Fig. 3B). T2 cells expressing the L^d restriction element (T2.L^d cells) were not recognized by *H-2*^{b}-restricted CTL lines (Fig. 3B). The K^b- and D^b-restricted HBsAg epitopes recognized by CTL have not yet been mapped. Nucleic acid vaccination thus revealed at least two class I-restricted CTL specificities of HBsAg in *H-2*^{b} mice that were not detectable following HBsAg protein immunization or by recombinant vaccinia-HBs virus infection.

TABLE 3. Serum antibody response to HBsAg in mice induced by particle or plasmid DNA immunization[a]

Cells	Serum antibody titer (mIU/ml)	
	BALB/c (*H-2*^{d})	C57BL/6 (*H-2*^{b})
Not primed	<20	<20
Primed with:		
HBsAg particles	387 ± 83	288 ± 165
Plasmid pRKS	2,230 ± 727	3,640 ± 814

[a] BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were immunized either intraperitoneally with 5 μg of HBsAg particles (without adjuvants) or intramuscularly with 100 μg of HBsAg-encoding pRKS DNA. Six weeks postinjection, the antibody response to HBsAg was determined in the serum as described previously (22). Mean values ± standard errors of the means for five mice are shown.

Serum antibody response to HBsAg in mice immunized by particles or plasmid DNA. BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were immunized either intraperitoneally with 5 μg of HBsAg particles (without adjuvants) or intramuscularly with 100 μg of HBsAg-encoding pRKS DNA. Six weeks postinjection, the antibody response to HBsAg was determined in the serum (22). Mice immunized with HBsAg particles as well as mice immunized by plasmid DNA developed readily detectable serological reactivity to HBsAg (Table 3). HBsAg-specific serum antibody titers were reproducibly higher in mice immunized with pRKS DNA (Table 3). High titers of antibodies to native HBsAg particles inducible by DNA immunization are explained by the observation that HBsAg is secreted from expressing cells. We have previously reported that boost injections with HBsAg particles or adsorption of HBsAg particles to alum increases the antibody response to HBsAg (21).

DISCUSSION

Nucleic acid immunization represents a simple and efficient technique to prime class I-restricted T cells. In this report, we demonstrate that DNA immunization can define an extended spectrum of potentially immunogenic epitopes of protein antigens that are not revealed by alternative vaccination protocols.

Class I-restricted CTL have been traditionally induced in mice by immunization with recombinant vaccinia viruses that express the corresponding protein of interest. The efficiency of this procedure has been interpreted in the light of the well-described requirement for the processing of endogenously synthesized protein antigens via a nonendosomal, intracellular pathway for the appropriate presentation of derivative antigenic peptides to class I-restricted CTL. This procedure has been successfully used for the induction of S_{28-39}-specific, L^d-restricted CTL in *H-2*^{d} mice as described here (see Results) and elsewhere (2, 14). We have recently shown, however, that nontraditional pathways can also induce $HBsAg_{28-39}$-specific CTL in *H-2*^{d} mice (20–23). Indeed, injection of exogenous HBsAg preparations without adjuvants by various routes is unexpectedly efficient in priming class I-restricted, murine CTL in vivo (20–22). In different presenting cell types, exogenously supplied native HBsAg particles efficiently enter an endosomal processing pathway for the generation of antigenic peptides for class I-restricted presentation (23). This processing pathway operates efficiently in peptide transporter-deficient T2 cells. Hence, the HBsAg system revealed a novel endosomal/lysosomal processing pathway for class I-restricted presentation of peptides derived from exogenous protein particles.

The type of HBsAg processing operating in the efficient class

I-restricted presentation of S epitopes after immunization with recombinant vaccinia-HBs virus or by intramuscular nucleic acid immunization is unresolved. Whereas the traditional non-endosomal endogenous antigen processing pathway is probably involved, the endosomal pathway for processing exogenous protein antigens for class I-restricted epitope presentation (23) may also play a role, since HBsAg expressed by cells after stable or transient expression of different transfected constructs is secreted. Whatever the mechanism, however, the data presented herein attest to the superiority of the DNA immunization strategy over the use of vaccinia virus and soluble HBsAg.

Our data indicate that nucleic acid immunization can be used effectively to define the immunogenicity of a protein antigen for class I-restricted T cells in vivo. Because of the efficiency of CTL induction by DNA immunization, this type of antigen delivery will be of interest not only for prevention but also for therapeutic vaccine strategies in which induction or boosting of specific CTL reactivity in chronically infected patients is the aim. Obvious candidates are vaccines designed to combat cancer and AIDS, as well as many other persistent viral infections, including chronic hepatitis B and C.

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