Antibody and Cytotoxic T-Cell Responses to Soluble Hepatitis B Virus (HBV) S Antigen in Mice: Implication for the Pathogenesis of HBV-Induced Hepatitis

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Immune responses to components of hepatitis B virus (HBV) are assumed to play an essential role not only in the elimination of the virus but also in the pathogenesis of HBV-induced hepatitis. Protective humoral immunity to HBV is mediated by immune responses to HBV surface antigen (HBsAg). It is important to know which HBsAg preparations induce which type of cellular and humoral immune responses under which immunization conditions. We studied in BALB/c mice the humoral (antibody) response and the class I-restricted cytotoxic T-lymphocyte (CTL) response to different preparations of HBsAg particles: recombinant, small protein particles; plasma-derived, mixed particles formed by large, medium, and small surface proteins; and different preparations of recombinant, mixed particles formed by large and small surface proteins. Specific antibody levels appeared in the sera of immunized mice 2 to 3 weeks after immunization and were correlated with the antigen dose used for priming. HBsAg-specific antibody levels were enhanced by boost injections or by adsorbing the antigen to aluminum hydroxide. Injected in particulate form without adjuvants in the dose range of 0.1 to 10 μg per mouse, all HBsAg preparations tested efficiently primed specific CD8+ CTL of defined restriction and epitope specificity. Specific CTL reactivity was detectable from 5 days to more than 4 months postimmunization. In the dose range tested, it was independent of the antigen dose used for immunization and not enhanced by repeated boost injections. CTL were not elicited by HBsAg adsorbed to aluminum hydroxide. We have thus defined conditions under which HBsAg induced preferentially either a cellular immune response or a humoral immune response. These findings may be relevant for the interpretation of HBV-associated immunopathologic phenomena.

Hepatitis B virus (HBV) surface antigens (HBsAg) are immunodominant antigens. The recombinant HBV small surface (S) protein adsorbed to aluminum hydroxide (alum) is the only recombinant vaccine now in widespread use to treat humans (8, 9). This vaccine has proven its effectiveness beyond doubt. Protection against HBV induced by this vaccine is correlated with high levels of serum antibodies specific for HBsAg (8, 9). It is unknown whether anti-HBsAg-specific, major histocompatibility complex (MHC) class I-restricted CD8+ cytotoxic T lymphocytes (CTL) are induced by this type of vaccination and whether anti-HBsAg-specific CTL are involved in the protective immune response against HBV. In HBsAg carriers persistently infected with HBV, anti-HBsAg-specific antibody levels are undetectable, and high levels of HBsAg particles are present in serum. Anti-HBsAg-specific CD8+ CTL have been isolated from some of these patients (17). It has been hypothesized that CD8+ CTL contribute to the immunopathology associated with acute or chronic HBV infection. CTL may destroy large numbers of HBV-infected hepatocytes (1, 25). Furthermore, CTL may specifically eliminate anti-HBsAg-specific B cells and hence reduce anti-HBsAg-specific B cells and hence reduce anti-HBsAg-specific antibody levels (2, 3, 10). It thus seems of interest to analyze in detail the immune reactivities stimulated by different HBsAg preparations by using different immunization protocols.

We have studied the humoral immune response and the CD8+ CTL response of BALB/c ($H-2^d$) mice to recombinant

HBsAg preparations. Recombinant S-protein particles have the unusual property that they efficiently prime a specific CTL response after a single injection of 0.1 to 10 μg of the antigen given without adjuvants. We show in the experimental system described that a small recombinant, viral protein of potential interest for human protective immunity and disease (HBV-induced hepatitis) can specifically stimulate in soluble form a CD8+ CTL response. Unexpectedly, a single immunization of mice with the same dose of S antigen adsorbed to alum elicited substantial antibody titers but failed to stimulate a CTL response. Different modes of immunization with HBsAg can thus stimulate preferentially either a humoral or a cellular immune response.

MATERIALS AND METHODS

Mice. BALB/cJ mice ($H-2^d L^{d+}$) and BALB/c $H-2^{dm2}$ ($H-2^d L^{d-}$) (dm2) mice were bred under specific-pathogen-free conditions in the animal colony of the Institute of Microbiology, Ulm University, Ulm, Germany. Male and female mice were used at 12 to 16 weeks of age.

Cells. The $H-2^d$ mastocytoma cell line P815 (TIB 64) and the $H-2^d$ B-lymphoma cell line A20 (TIB 208) were obtained from the American Type Culture Collection (Rockville, Md.).

Recombinant S antigen. The S-protein (HBsAg) sequence, subtype ad, was inserted into the *Hansenula polymorpha* expression vector pMPT121 and transformed into *H. polymorpha* host strain RB10 (12). Yeast transformants efficiently expressing S protein 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.

* Corresponding author. Mailing address: Institute of Medical Microbiology and Immunology, University of Ulm, Albert-Einstein-Allee 11, D-89069 Ulm, Germany. Phone: 49 731 502 4490/3362.

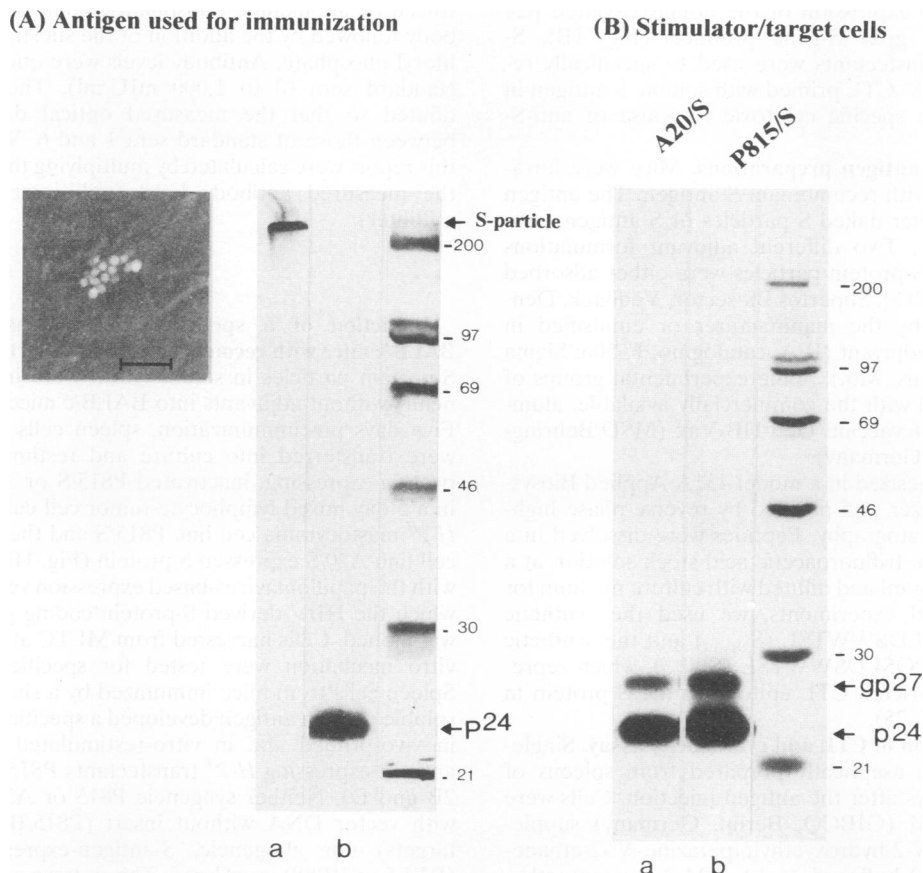**(A) Antigen used for immunization**   **(B) Stimulator/target cells**

FIG. 1. (A) Characterization of the HBV S antigen used for immunization. Purified (yeast-expressed) S-antigen particles were directly analyzed by SDS-PAGE (lane a) or were completely disrupted by boiling for 5 min in 2% SDS–5% β-mercaptoethanol before SDS-PAGE analysis. S antigen was detected by Western blotting. Phosphotungstic staining of the S-antigen preparations revealed their particle structure in electron microscopy (bar, 100 nm). (B) Expression of HBV S antigen in transfected murine H-2^d stimulator/target cells. Mastocytoma P815 cells and B-lymphoma A20 cells were transfected with DNA of the expression vector BMG/HBS into which the S gene had been cloned. G418-resistant transfectants expressed the nonglycosylated p24 S protein and the glycosylated gp27 glycoprotein, shown by labeling the cells with [^{35}S]methionine, extraction, immunoprecipitation for S antigen, and analysis by SDS-PAGE. Sizes are indicated in kilodaltons.

S-antigen particles were purified by adsorption to silica gel, column chromatography, and isopycnic ultracentrifugation as described previously (12, 16). Analysis of the purified S antigen by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting) by using polyclonal rabbit anti-S-antigen antibodies (lot no. 61WN003; Behringwerke, Marburg, Germany) and ^{35}S-labeled protein A as described previously (32) revealed a homogeneous band with an apparent molecular mass of 24 kDa expected for the nonglycosylated form of the S antigen (Fig. 1A). Preparations of recombinant S-protein particles extensively dialyzed to remove peptides revealed no evidence of contaminating S-protein monomers, dimers, or proteolytic fragments (Fig. 1A). The S protein used for immunization self-assembled into typical 20-nm particles, as demonstrated in electron microscopic examinations of the antigen preparation (Fig. 1A).

Recombinant mixed S/L particles, composed of S and large (L) HBV surface proteins (at a ratio of 1:15), were produced in *H. polymorpha* as described previously (16). S/ΔL particles, containing S proteins and a truncated variant of the L surface protein (5), were kindly provided by M. de Wilde (Smith Kline Biologicals, Rixensart, Belgium).

S-protein-expressing murine transfectants. The bovine pap-

illomavirus-based vector BMGNeo (18) (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. The *Xho*I-*Bgl*II fragment of HBV, subtype ayw (11), from plasmid TKTHBV2 (a generous gift from M. Meyer, Munich, Germany), encoding the S-antigen sequence up to the poly(A) signal, was cloned into the BMGNeo vector.

Two murine H-2^d cell lines, the mastocytoma cell line P815 and the B-lymphoma cell line A20, were transfected with vector DNA which either contained or did not contain the S-antigen-encoding HBV gene. Cloned and subcloned G418-resistant lines were analyzed. Cells (10^7) from selected clones were characterized for S-antigen expression by labeling with [^{35}S]methionine as described previously (32). After labeling, cells were washed with phosphate-buffered saline and extracted with lysis buffer (120 mM NaCl, 0.5% Nonidet P-40, 10% immunoglobin-free fetal calf serum [about 10 mg of protein per ml], 50 mM Tris-hydrochloride [pH 8.0]) for 30 min at 4°C. Extracts, cleared by centrifugation and incubated with protein A-Sepharose, were subsequently immunoprecipitated for S antigen by using rabbit anti-S serum and protein A-Sepharose. Cell lines transfected with vector constructs containing the S-antigen-encoding gene (i.e., P815/S and

A20/S) showed stable expression of the nonglycosylated p24 and the glycosylated gp27 S-gene products (Fig. 1B). S-antigen-expressing transfectants were used to specifically restimulate in vitro CD8^{+} CTL primed with soluble S antigen in vivo and to read the specific cytotoxic response of anti-S-antigen-specific CTL.

Immunization and antigen preparations. Mice were intraperitoneally injected with recombinant S antigen. The antigen preparations were either naked S particles or S antigen associated with adjuvants. Two different adjuvant formulations were tested. Purified S-protein particles were either adsorbed to alum (Alhydrogel [2%]; Superfos Biosector, Vedbaek, Denmark) as instructed by the manufacturer or emulsified in incomplete Freund's adjuvant (IFA; catalog no. F5506; Sigma Chemical Co., St. Louis, Mo.). Some experimental groups of mice were immunized with the commercially available, alum-adsorbed recombinant vaccine Gen-HB-Vax (MSD/Behringwerke AG, Marburg, Germany).

Peptides were synthesized in a model 431A Applied Biosystems peptide synthesizer and purified by reverse-phase high-pressure liquid chromatography. Peptides were dissolved in a 70% acetonitrile–0.1% trifluoroacetic acid stock solution at a concentration of 10 mg/ml and diluted with culture medium for use. In the described experiments, we used the synthetic 12-mer peptide IPQSLDSWWTSL (S_{28-39}) and the synthetic 15-mer peptide ILTIPQSLDSWWTSL (S_{25-39}), which represent a defined L^d-restricted CTL epitope of the S protein in $H-2^d$ BALB/c mice (1, 25).

In vitro restimulation of CTL and cytotoxicity assay. Single-cell suspensions were aseptically prepared from spleens of immunized mice 6 days after the antigen injection. Cells were suspended in α-MEM (GIBCO, Berlin, Germany) 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). To the culture medium was further added 5% (vol/vol) of a selected batch of concanavalin A-stimulated rat spleen cell supernatant containing interleukin-2 (29). Responder cells (3×10^7) were cocultured with 1.5 $\times 10^6$ syngeneic, S-antigen-expressing transfectants (irradiated with 20,000 rad) in 10 ml of medium in upright 25-cm^2 tissue culture flasks in a humidified atmosphere with 7% CO_2 at 37°C.

Cytotoxic effector populations were harvested after 5 to 7 days 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 different targets. After a 4-h incubation at 37°C, 100 μl of supernatant was collected for gamma counting. The percentage 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 20% of the mean.

Determination of serum antibody levels. Antibodies against the HBV S antigen were detected in mouse sera by using the commercially available test IMxAUSAB (Abbott, Wiesbaden, Germany). In this readout, microparticles coated with HBsAg were mixed with mouse serum samples to allow specific binding of anti-S-antigen antibodies. Microparticles coated with antigen-antibody complexes were transferred and irreversibly bound to a glass fiber matrix. Biotinylated recombinant S antigen was added, and binding to the complex was traced by an alkaline phosphatase-conjugated antibiotin antibody followed by the addition of the substrate 4-methylumbelliferyl phosphate. 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-international units per milliliter).

RESULTS

Induction of a specific CTL response by immunizing BALB/c mice with recombinant HBV S particles. Recombinant S-protein particles in soluble form were injected intraperitoneally without adjuvants into BALB/c mice (1 μg per mouse). Five days postimmunization, spleen cells from primed mice were transferred into culture and restimulated with the S-protein-expressing, inactivated P815/S or A20/S transfectants in a 5-day mixed lymphocyte-tumor cell culture (MLTC). The $H-2^d$ mastocytoma cell line P815/S and the $H-2^d$ B-lymphoma cell line A20/S expressed S protein (Fig. 1B) after transfection with the papillomavirus-based expression vector BMGNeo into which the HBV-derived S-protein-coding gene (subtype ayw) was cloned. Cells harvested from MLTC after 5 to 6 days of in vitro incubation were tested for specific cytotoxic activity. Spleen cells from mice immunized by a single injection of this soluble protein antigen developed a specific CTL response: the in vivo-primed and in vitro-restimulated cells lysed the S-antigen-expressing $H-2^d$ transfectants P815/S and A20/S (Fig. 2B and D). Neither syngeneic P815 or A20 cells transfected with vector DNA without insert (P815/BMG or A20/BMG targets) nor allogeneic, S-antigen-expressing transfectants (RBL5 or BB88) were lysed. This pattern of lysis was observed irrespective of the P815/S or A20/S stimulator cell used for specific restimulation in the MLTC in vitro (Fig. 2B and D). Restimulation of cells with inactivated P815/BMG or A20/BMG transfectants (which do not express S antigen) in the MLTC did not restimulate a CTL response in vitro (Fig. 2C). Anti-S-antigen-specific CTL responses were not observed in primary MLTC, in which lymph node or spleen responder cells from unprimed BALB/c mice were cocultured with A20/S or P815/S transfectants in the presence of conditioned medium (Fig. 2A). In vivo priming of CTL was thus essential for the induction of a specific cytolytic response. Methods of priming of mice with S particles by the subcutaneous, intraperitoneal, and intravenous routes were equally efficient (data not shown). Comparable levels of specific CTL reactivity were found in mice immunized with 100 ng, 1 μg, or 10 μg of the antigen (Table 1). A boost injection with S-antigen particles was not required to reveal this response (Table 1). Effector cells of this response expressed the CD3^{+} CD4^{−} CD8^{+} phenotype because they were eliminated by treatment with anti-CD8 antibody plus complement but were not affected by treatment with anti-CD4 antibody plus complement (Table 2). BALB/c-derived CTL lysed S-antigen-expressing $H-2^d$ MHC class I[+] class II[−] P815/S targets and class I[+] class II[+] A20 targets equally well (Fig. 2B and D). An anti-L^d-specific monoclonal antibody blocked lysis of P815/S targets by primed CTL (data not shown). Immunization of $H-2^d$ L^{d+} BALB/c but not the congenic $H-2^d$ L^{d-} BALB/c dm2 mice stimulated an S-specific CTL response (Table 3). These data indicate that all S-antigen-specific CD8^{+} CTL from BALB/c mice are L^d restricted (1, 25). CTL primed by S-protein particles recognized an epitope located between residues 28 and 39 of the S protein (Fig. 2B and D), confirming the mapping of the CD8^{+} CTL-defined
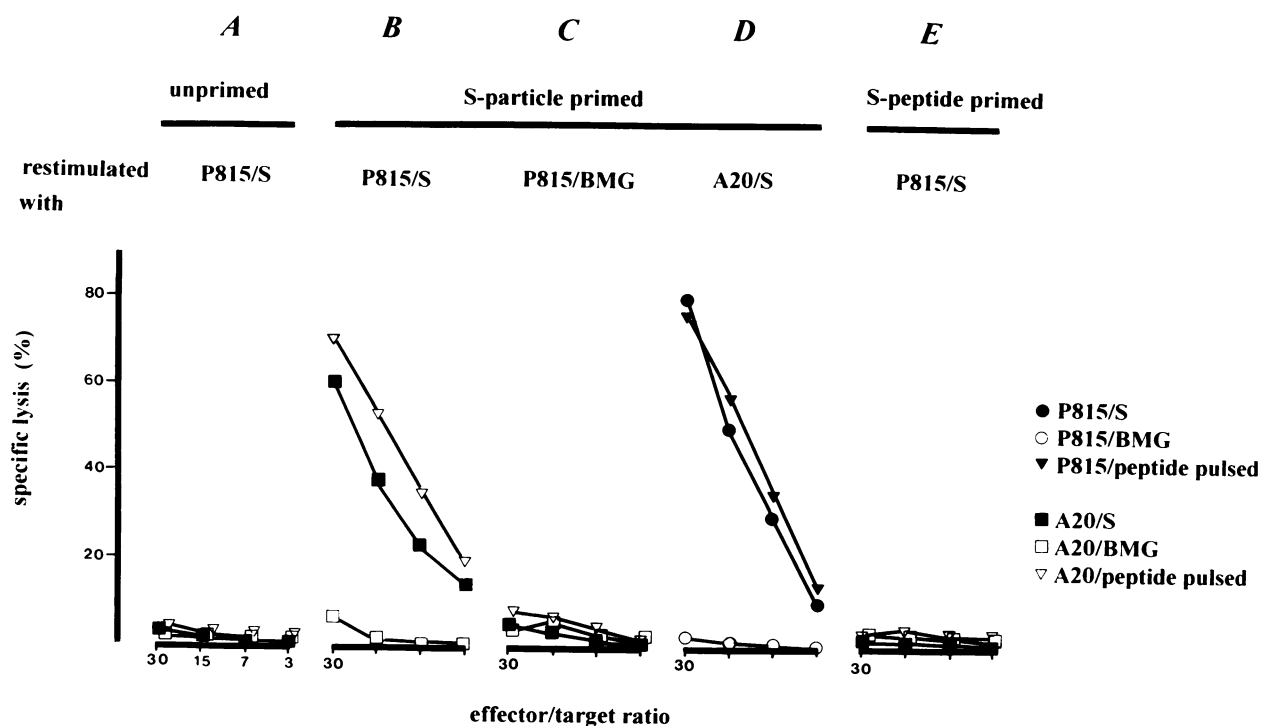
FIG. 2. S-protein particles, but not S-protein-derived peptides, prime CTL from BALB/c mice. BALB/cJ mice (H-2^d L^{d+}) were primed by a single injection of either 1 μg of S-protein particles (B to D) or 1 μg of the S_{28-39} synthetic 12-mer S peptide (E), without adjuvants. Lymphoid cells prepared from immunized mice 6 days postimmunization were cocultured with syngeneic, S-antigen-expressing P815/S or A20/S transfectants (irradiated with 20,000 rad). A control group included unprimed BALB/c cells stimulated in vitro with inactivated P815/S (A). Cytotoxic effector populations were harvested after 5 days of in vitro culture. The cytotoxic response was read against the S-antigen-expressing targets P815/S and A20/S and the negative control targets A20/BMG and P815/BMG (transfected with BMGNeo vector DNA) in a standard ^{51}Cr release assay. In addition, A20 or P815 cells pulsed for 1 h with 10^{-8} M S_{28-39} peptide were used as targets. Data shown are means of triplicate cultures. The standard error of the mean of triplicate data was always less than 15% of the mean.

epitopes performed in BALB/c mice infected with S-antigen-expressing, recombinant vaccinia virus (1).

CTL are primed by different preparations of naked S-antigen particles. A specific CTL response of BALB/c mice to S antigen was induced with comparable efficiency with S particles, with S/L particles, or with S/ΔL particles (Table 1). Plasma-derived S particles composed of S, M, and L surface

proteins were also very efficient in eliciting a CTL response in BALB/c mice (data not shown). The S-specific CTL reactivity was detectable for at least 3 months after the antigen injection. Boost injections with the same dose of the antigen used for priming (administered 3 to 15 weeks after the first injection) did not enhance the specific CTL reactivity (Table 1). We excluded the possibility that the partial proteolytic degradation of S protein after its injection into the mouse yielded extracel-

TABLE 1. Stimulation of CTL in BALB/c mice immunized by
different preparations of naked S particles but not
by S particles adsorbed to alum

Antigen prepn (μg/injection)	Specific cytotoxic reactivity (% specific release)[a]			
	Naked antigen		Antigen adsorbed to alum	
	Primary response	Boosted response	Primary response	Boosted response
S particles (0.1)	52	65	NT	NT
S particles (1)	76	68	<3	<3
S particles (10)	52	65	<5	<6
S/L particles (2)	81	74	<3	<5
S/ΔL particles (2)	68	52	<2	<5
Gen-HB-Vax (2)	NT	NT	<3	<2
12-mer S peptide (1)	<3	<4	<6	<5
15-mer S peptide (1)	<5	<5	<4	<6

[a] The specific cytotoxic reactivity of in vivo-primed and in vitro-restimulated cells was read against syngeneic P815/S transfectants. The effector/target ratio was 20:1. NT, not tested.

TABLE 2. Mediation by CD8+ CTL of the L^d-restricted response
to soluble S-protein particles in H-2^d mice

Group[a]	% Specific lysis[b]	
	P815/S	P815/BMG
1	47	2
2	44	4
3	42	4
4	8	3

[a] Spleen cells were obtained from BALB/c mice primed 7 days previously by an intraperitoneal injection of 1 μg of soluble S-protein particles. Primed cells were not treated (group 1) or were treated with low-toxicity rabbit complement (Cedarlane catalog no. CL3051), complement (group 2), anti-CD4 (monoclonal antibody GK1.5) plus complement (group 3), or anti-CD8 (monoclonal antibody 53-6.7) plus complement (group 4). Treated cells were washed twice, resuspended in culture medium, and restimulated for 6 days with inactivated A20/S transfectants.

[b] The cytotoxic response was read against H-2^d S-antigen-expressing P815/S transfectants or P815 targets transfected with the vector without insert (P815/BMG). The effector/target ratio was 20:1.

TABLE 3. L^d restriction of anti-S-protein-specific CD8$^+$ CTL

H-2^d mouse strain[a]	Expt	% Specific lysis[b]	
		P815/S	P815/BMG
BALB/c	1	52	1
	2	61	3
dm2	1	2	1
	2	5	4

[a] BALB/cJ mice (H-2^d L^{d+}) or mutant BALB/c $dm2$ mice (H-2^d/L^{d-}) were immunized by an intraperitoneal injection of 1 μg of S-protein particles without adjuvants. Six days postimmunization, spleen cells were transferred into culture and restimulated with inactivated A20/S transfectants. After 5 days in the MLTC, the cytolytic reactivity of in vivo-primed and in vitro-restimulated cells was tested.

[b] See Table 2, footnote b.

lular, immunogenic peptides that stimulated a class I-restricted CTL response without any requirement for processing of this protein antigen. The synthetic, S-derived 12-mer IPQSLDSW WTSL and 15-mer ILTIPQSLDSWWTSL peptides (representing the L^d-restricted CTL epitope of HBV S protein) sensitized P815 cells in vitro for lysis by CTL primed in vivo to S-protein particles (Fig. 2B and D). Immunization of BALB/c mice with these synthetic 12-mer and 15-mer peptides without adjuvants, adsorbed to alum (Table 1) or emulsified in IFA (data not shown), did not prime CTL (Fig. 2E; Table 1). Hence, S antigen has to be processed in vivo in order to be immunogenic for CD8$^+$ CTL.

CTL are not primed by S-antigen particles adsorbed to alum or emulsified in mineral oil. We tested the influence of the adjuvants alum and IFA on the specific CTL induction by soluble S protein. BALB/c mice were immunized with recom-

binant S-antigen particles adsorbed to alum or emulsified in IFA. In contrast to immunizations with naked S-antigen particles that efficiently primed CTL, the alum-adsorbed S antigen did not stimulate a CTL response in vivo (Table 1). This was found in six independent experiments in which we tested the different S-antigen preparations adsorbed to Alhydrogel and the commercial vaccine preparation (Table 1). Similar data were obtained in immunization experiments using IFA-emulsified S-antigen preparations (data not shown). Additional boost injections with alum-adsorbed or IFA-emulsified S antigen 3 to 15 weeks after the first injection did not reveal evidence for an induction of an anti-S CTL response (Table 1). The data indicate that naked S-antigen particles efficiently primed CTL in vivo, but the same type and dose of the antigen adsorbed to alum or emulsified in mineral oil (IFA) did not stimulate CTL in vivo.

The specific antibody response of BALB/c mice to recombinant S-particles. Three weeks postinjection, the levels of antibody specific for S antigen in the sera of immunized mice were determined (Fig. 3). Animals immunized with 1 to 3 μg of S or S/L particles (without adjuvants) developed only low levels of S-specific antibodies, while animals immunized with a single injection of 10 μg of S particles or 3 μg of human plasma-derived HBsAg without adjuvants showed readily detectable serological reactivity to HBV S antigen in the serum. Serum antibody levels increased 10^2- to 10^3-fold following a boost injection with the same preparation and the same dose of S antigen without adjuvants.

Compared with the primary and boosted antibody responses of mice immunized with naked S particles without adjuvants, mice immunized and boosted with S-protein preparations adsorbed to alum (or emulsified in IFA; data not shown) showed enhanced serum antibody levels. In all mice immu-
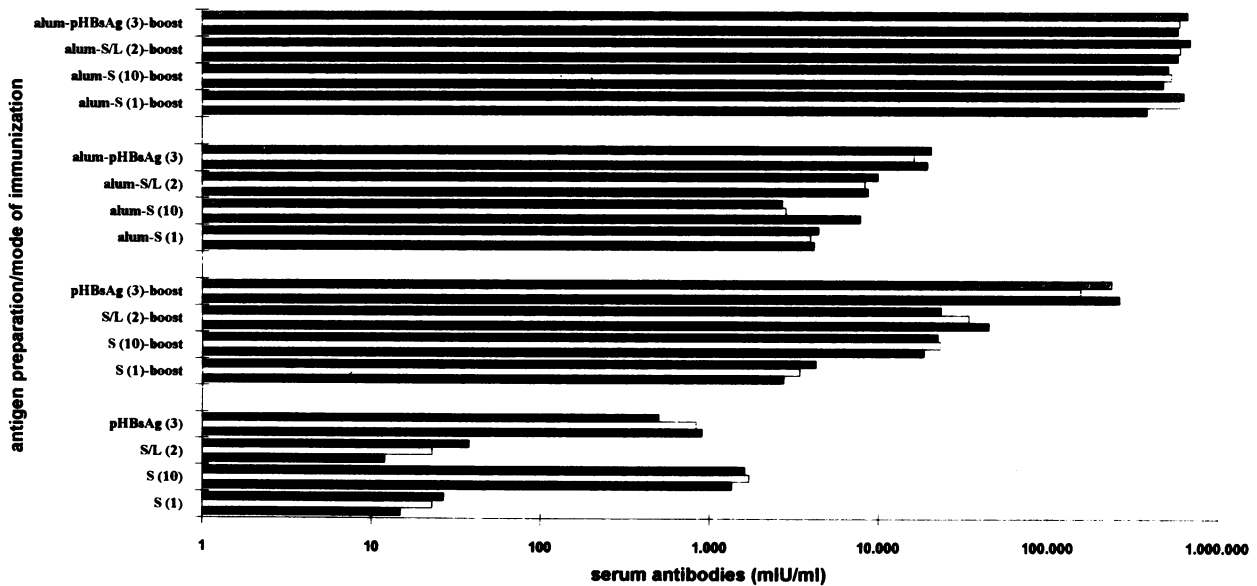

FIG. 3. Specific antibody response of BALB/c mice immunized with either naked S-antigen particles or alum-adsorbed S-antigen particles. BALB/c mice were immunized intraperitoneally with different S-antigen preparations, using the doses (micrograms per injection) indicated in parentheses. Specific antibody levels were measured in the sera of immunized mice 3 weeks after the priming injection and 3 weeks after a boost injection. The same dose of antigen was used for the priming injection and for the boost injection. Mice were immunized with S antigen either in the form of naked particles (bottom eight groups) or adsorbed to alum (upper eight groups). The antigen preparations tested were recombinant, yeast-expressed S particles, S/L particles, and purified human plasma-derived HBsAg (pHBsAg). The values for three individual mice in each group are shown.
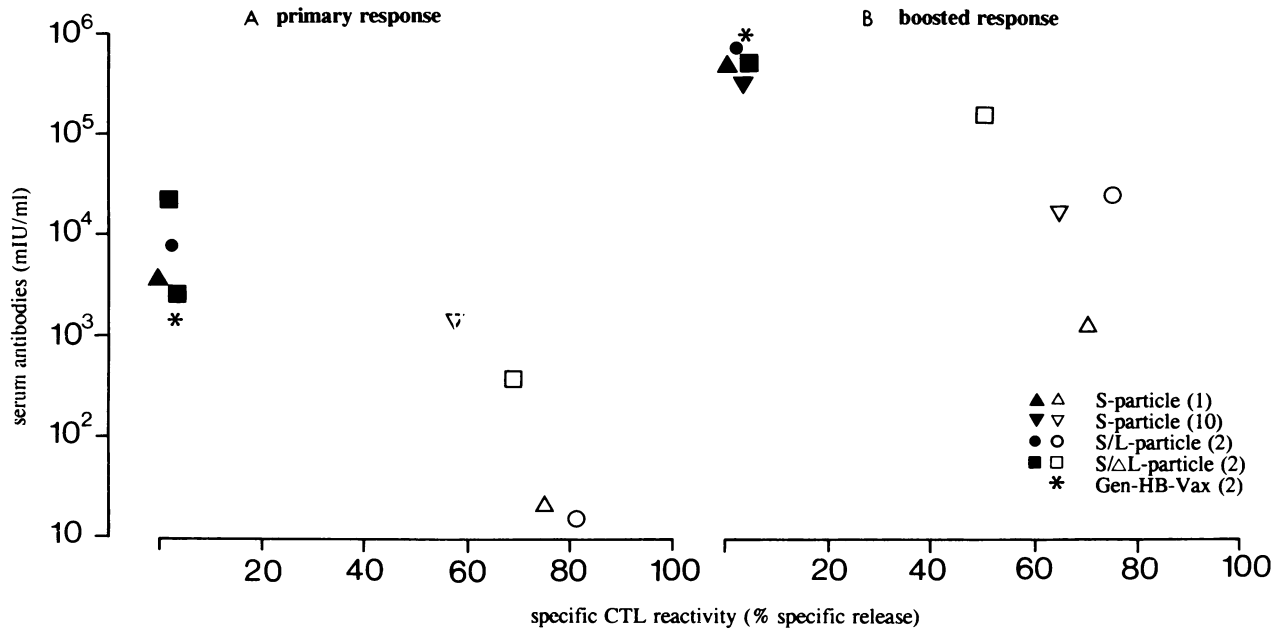
FIG. 4. Antibody and CTL responses of BALB/c mice to different preparations of HBV S antigen. BALB/c mice were immunized intraperitoneally with different S-antigen preparations, using the doses (micrograms per injection) indicated in parentheses. Specific serum antibody levels and specific cytotoxic reactivity were measured in immunized mice 3 weeks after the priming injection (A) and 3 weeks after a boost injection (B). Mice were immunized with S-antigen preparations either in the form of naked particles (open symbols) or adsorbed to alum (closed symbols). The antigen preparations tested were recombinant, yeast-expressed S, S/L, and S/ΔL particles and alum-adsorbed S antigen in the form of the commercial vaccine Gen-HB-Vax. Each value represents the mean antibody level and the mean specific cytotoxic reactivity of three individual mice in each experimental group.

nized with S antigen plus adjuvants, the specific anti-S antibody levels in serum were 10- to 10^3-fold higher in primary and in boosted responses compared with serum antibody levels detectable in mice immunized with S-antigen without adjuvants (Fig. 3).

Comparison of primary and boosted responses of CD8⁺ CTL and B cells of BALB/c mice with responses to HBV S antigen administered with or without adjuvants. The CD8⁺ CTL reactivity of BALB/c mice to soluble S-protein particles was detectable as early as 5 days after the immunization and persisted for many months. It was independent of the antigen dose used for immunization in the range of 0.1 to 10 μg of antigen per mouse and was not enhanced by repeated boost injections. It was not elicited when mice were immunized with S particles adsorbed to alum or emulsified in mineral oil (Fig. 4).

The humoral (B-cell) response of BALB/c mice to S protein measured in terms of specific antibody levels in serum appeared 2 to 3 weeks after immunization. It was correlated with the antigen dose used for priming and was enhanced by boost injections and by immunizing mice with S particles adsorbed to alum or emulsified in mineral oil (Fig. 4).

DISCUSSION

Using different preparations of the 226-amino-acid viral surface protein HBsAg, we elicited humoral immune responses as well as CD8⁺ CTL responses in BALB/c mice. The mode of immunization critically influenced the pattern of immune effector functions specifically recruited into the response. The phenomenology of the cellular and humoral immune responses of BALB/c mice to HBsAg differed in terms of kinetics of appearance, dose dependency of the responses,

influence of boost injections, and influence of adjuvant formulations.

The CTL response induced by recombinant S-protein particles. This report describes a novel aspect of the immunogenicity of the HBV S antigen for T cells: naked S-antigen particles injected by different routes (subcutaneously, intravenously, and intraperitoneally) into BALB/c mice efficiently primed anti-S-antigen-specific CD8⁺ CTL. It is not known why naked S-antigen particles are so efficient in priming CTL in vivo. The general rule seems to be that soluble protein antigens enter antigen-presenting cells (APC) by endocytosis, are processed via the exogenous pathway in a late endosomal compartment, are presented on the surface of APC in the context of MHC class II glycoproteins, and selectively stimulate CD4⁺ T cells (reviewed in references 4 and 21). In contrast, CD8⁺ CTL usually recognize peptides derived from intracellularly synthesized and processed endogenous protein antigens that are presented on the cell surface in association with MHC class I molecules (reviewed in references 13, 35, and 40). Some exceptions to this rule have been published recently. We reported that the injection of recombinant large T antigen of the simian virus 40 into C57BL/6 (H-2^b) mice efficiently primed CD8⁺ CTL in vivo (31, 32). Intraperitoneal, subcutaneous, or intramuscular injection into BALB/c mice of a single dose of 10 μg of purified, soluble influenza virus nucleoprotein (NP) without adjuvants stimulated anti-NP-specific CTL responses (33, 37). Influenza virus hemagglutinin-specific CD8⁺ CTL were induced by soluble, recombinant protein produced in *Escherichia coli* (39). The poliovirus VP1 capsid protein apparently entered the endogenous processing pathway in vivo and in vitro after immunization of BALB/c mice with 20 μg of the soluble form of this structural viral protein (20). Some

covalent modifications of protein antigens such as lipid conjugation facilitate their access to the endogenous processing pathway and their stimulation of a CD8$^+$ CTL response in vivo (7, 24, 30). Injected in soluble, native form, some viral proteins can thus be processed by the endogenous pathway in vivo and in vitro. Very little is known about the cell biology of this type of processing by APC.

The influence of alum and mineral oil adjuvants on CTL priming by protein antigens. S-antigen particles either with or without the adjuvant alum or mineral oil did not stimulate a CTL response in BALB/c mice. This might result from particular properties of either the S antigen or the two adjuvants (alum and IFA) tested. Inefficient or absent CTL priming to antigen in alum or mineral oil may be a property of particulate protein antigens because it has also been observed with two different types of viral nucleoprotein particles: the inclusion of mineral oil or alum in the formulation inhibited the induction of CTL to human immunodeficiency virus type 1 Gag particles (14) or the Ty-VLP vaccine (22). It is not known whether alum- or mineral oil-based adjuvants prevent access of these particulate protein antigens to the endogenous processing pathway of APC or whether these adjuvants induce a type of cellular immune response in which the expansion of an induced CD8$^+$ CTL response is downregulated to undetectable levels. In contrast, some reports indicate that antigens with mineral oil as an adjuvant can induce a CTL response. Sendai virus nucleoprotein-derived peptides with mineral oil as an adjuvant induced murine CTL responses (19). Other types of adjuvants, such as ISCOM (26, 34, 36), saponins (28, 38), or liposomes (6, 23, 27), facilitate in vivo priming of CD8$^+$ CTL to protein antigens. Some protein antigens emulsified in complete Freund's adjuvant apparently stimulate a CTL response. Poliovirus VP1 or Sendai virus-derived immunogenic peptides emulsified in complete Freund's and injected in mice stimulated *H-2* class I-restricted CD8$^+$ CTL response (20). Adjuvants have been extensively used to augment immune responses to antigen. They will be increasingly important to realize the promises of recombinant proteins and peptides for use as synthetic subunit vaccines. Alum is the only adjuvant currently authorized for human use. The data reported in this paper stress the importance of defining the role of adjuvants in favoring sensitization of certain selected lymphocyte subsets at the expense of others. In addition to augmenting B-cell responses, many adjuvants seem to change the pattern of T- and/or B-cell subsets stimulated in response to antigen. It has become obvious in many experimental models that different T-cell subsets play distinct disease-preventing or disease-promoting roles during a particular infection. An essential prerequisite for vaccine development is to find an appropriate way to deliver the antigen so that it will stimulate the right type of protective response and will avoid the induction of undesirable or even dangerous immunopathological reactions.

The anti-HBsAg immune reactivity in HBV-induced liver pathology. For many years, effective methods for the virological diagnosis of HBV infections have been established, and the diagnostic value of markers that correlate with the course of HBV infection and HBV-induced disease has been established (reviewed in reference 15). In contrast, very little is known about the factors that determine the very different clinical course of HBV infection in patients. Three different courses of the disease are observed in HBV-infected adults. (i) About 25% of HBV-infected adults will suffer from acute hepatitis. Of these, about 1% will die from fulminant hepatitis with liver dystrophy, and 99% will recover with production of anti-HBsAg antibodies and lifelong immunity. (ii) About 65% of HBV-infected adults have a subclinical infection in the

course of which HBV is eliminated and stable postinfectious immunity develops. (iii) About 10% of HBV-infected adults will not eliminate the virus, will not develop anti-HBsAg antibodies detectable in serum, but will have high levels of HBV-derived HBsAg particles present in serum. Of these chronically HBV-infected patients, 10 to 30% will develop chronic liver disease (hepatitis, cirrhosis, carcinoma), whereas 70 to 90% will be healthy HBsAg carriers without evidence of hepatitis. The rate of development of chronic disease is influenced by the age at the time of infection and may be as high as 90% in connatal HBV infections. HBV infection is not cytolytic for hepatocytes. Hence, the destruction of hepatocytes in the course of hepatitis does not seem to result from a direct cytopathic effect of HBV but may be induced by specific CTL attack. This interpretation is compatible with the observation that patients with fulminant, lethal hepatitis have very low or undetectable levels of viremia. The available data do not indicate whether the protective immune response (mediating HBV clearance) and the disease-promoting immune response (inducing hepatitis) are mediated by different immune effector functions or by different magnitudes of the same immune effector mechanism. We have shown in this report that the same viral protein antigen (HBsAg) can stimulate very different immune reactivities in an inbred mouse strain (BALB/c), depending on the mode of its delivery. Interestingly, the specific stimulation of CTL (which might be involved in the induction of hepatitis) was undetectable with use of the immunization protocol established for widespread human vaccination. Hence, the type of anti-HBV vaccine chosen might have been a very fortunate choice.

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