ELSEVIER

Vaccine 17 (1999) 1035–1041

Vaccine

Enhanced immunogenicity of hepatitis B surface antigen by insertion of a helper T cell epitope from tetanus toxoid

Murty V. Chengalvala *, Ramesh A. Bhat, Bheem M. Bhat, Steven K. Vernon, Michael D. Lubeck

Discovery Research, Wyeth Ayerst Research, P.O. Box 8299, Philadelphia, PA 19101, USA

Received 26 September 1997; received in revised form 12 August 1998; accepted 12 August 1998

Abstract

The currently marketed hepatitis B vaccines in the U.S. are based on the recombinant major hepatitis B surface antigen (HBsAg) of hepatitis B virus. Although a large majority of individuals develop protective immunity to HBV-induced disease after three immunizations, routinely a small but a significant percentage of the human population does not respond well to these vaccines. In this report, we describe the generation of a novel HBsAg molecule containing a T_h epitope derived from tetanus toxoid (TT). Using recombinant DNA technology, the TT T_h epitope (TTe) was inserted into the HBsAg coding sequence. Using a recombinant adenovirus expression system, HBsAg–TTe chimeric protein was produced in A549 cells and found to be secreted into culture medium as 22 nm particles. The chimeric HBsAg particles were readily purified by immunoaffinity chromatography and their immunogenicity was evaluated relative to native HBsAg produced in an adenovirus expression system. When evaluated in inbred and outbred strains of mice, HBsAg–TTe was shown to enhance several-fold the anti-HBs response relative to native HBsAg. Further enhanced responses were observed in mice primed with TT. This highly immunogenic form of HBsAg has promise as an improved HBsAg subunit vaccine.

Keywords: T cell-B cell collaboration; Hepatitis B; Epitopic Suppression; Vaccination

1. Introduction

Hepatitis B virus (HBV) causes significant health problems worldwide and chronic carriers of this virus often die from active hepatitis and liver cirrhosis. HBV is involved in chronic hepatocellular carcinoma and is one of the few cancers known to be caused by a virus [1]. The envelope of HBV particles consists of surface antigen protein of three different sizes: small (S), middle (S + pre-S2) and large (S + preS2 + preS1). Recombinant HBV subunit vaccines are available and these are made of HBsAg particles consisting of S antigen. HBsAg is a relatively weak immunogen in humans that requires three sequential injections to elicit long lasting humoral responses and, in general, anti-HBs antibody responses that exceed 10 mIU are indicative of immunity to hepatitis B infection [2–4]. Furthermore, a small proportion (about 5–10%) of normal vaccine recipients and about 40–50% of patients on maintenance hemodialysis with depressed immune responses do not respond well to these vaccines [5, 6]. Other hypo- and non-responders include alcoholics [7, 8], homosexual men and HIV-infected individuals [9]. HBV envelope proteins have been shown to contain three epitopes that can induce protective antibodies. The major epitope, termed 'a' epitope is discontinuous, involving both quaternary and tertiary structure of the S protein and both humans and mice show a dominant response to this antigen [10] whereas the other two are continuous epitopes located in the N-terminal parts of the preS2 and preS1 regions. Inclusion of preS sequences in the HBsAg molecule helped to circumvent the non-responsiveness to the S region in low responder mice but failed to enhance immunogenicity of HBV vaccine in humans [11].

* Corresponding author. Tel.: 610-341-2617; fax: 610-989-4832.

0264-410X/99/$19.00
PII: S0264-410X(98)00318-1

Some poorly immunogenic polypeptides have shown enhanced immunogenicity upon coupling to carrier molecules which contain strong T_h epitopes. Unfortunately, this approach has been shown to suffer from both a lack of proven carrier molecules suitable for human use and from the phenomenon of specific epitopic suppression [12]. Epitopic suppression occurs when the priming of a host with carrier results in a diminished anti-peptide response following subsequent immunization with the peptide linked to carrier [12, 13]. Studies in the TT antigen system have suggested that such suppression may be mediated by carrier-specific B cells and suppressor T cells [14, 15]. Schutze et al. [12] have further demonstrated that hapten-specific B cells fail to collaborate effectively with carrier-specific T cells, perhaps due to antigenic competition through clonal dominance. For certain carrier proteins individual epitopes recognized by T suppressor cells, B cells or T_h cells may be physically distinct (non-overlapping) allowing researchers to identify peptides from these carriers that exhibit T_h but not suppressor function [16–18]. For example, Etlinger et al. [19] have identified a TT peptide bearing a T_h cell epitope (amino acids 73–99) that is non-reactive with antisera against TT, indicating a potential lack of B cell reactivity and a possible separation of the T_h epitope from B cell mediated suppressor activity. Furthermore, it was also shown that linking this T_h epitope to a B cell epitope peptide from the major surface protein of *Plasmodium falciparum* enhanced the antibody response to this peptide by several fold, demonstrating that this TT T_h epitope exhibits carrier function [19]. Importantly, priming with TT prior to immunization with the fusion peptide did not stimulate cell-mediated immune suppression to the *P. falciparum* surface protein [19].

In this study we have examined the effect of introducing a T cell helper epitope of tetanus toxin into HBsAg and have shown a significant increase in the immunogenicity of the chimeric protein compared to native HBsAg in both inbred and outbred mice. Moreover, priming of mice with TT further increased subsequent booster responses to the chimeric protein.

2. Materials and methods

2.1. Expression of chimeric HBsAg

The Wyeth–Ayerst Ad7 vaccine strain (55142) was engineered to express the chimeric HBsAg protein. A recombinant virus, designated Ad7ΔE3H–TTe, was generated by deleting 8 map units (mu) in the E3 region (between 80–88 mu) and by inserting a synthetic Ad7 tripartite leader followed by the coding sequences of the major S HBsAg gene containing the TTe sequence (corresponding to amino acids 73–99 of TT) [20]. The expressed chimeric protein is under the control of E3 promoter [21]. A recombinant Ad7 expressing native HBsAg without the TTe insert, Ad7ΔE3H, was previously described [21, 22] and used for preparation of HBsAg used in control animals.

2.2. Purification of chimeric HBsAg

Confluent A549 cell (human lung carcinoma) monolayers were inoculated with Ad7ΔE3H or Ad7ΔE3H–TTe, culture medium was collected at 72 h post-inoculation and concentrated by ultrafiltration. HBsAg was purified by immunoaffinity column chromatography using a monoclonal antibody to HBsAg (A5c11, Centocor, Malvern, PA) immobilized to CNBr activated Sepharose 4B (Pharmacia, Sweden). Concentrated medium from infected cultures was passed over the affinity column and, after washing off unbound protein, the specifically retained protein was eluted with 5 M potassium iodide. HBsAg content was quantitated by a radioimmunoassay (AUSRIA kit, Abbott Laboratories, Chicago, IL) after removing the potassium iodide by dialysis.

2.3. Electron microscopic examination of HBsAg–TTe particles

Electron microscopy was performed as described earlier [23]. Briefly, an aliquot of immunoaffinity purified HBsAg–TTe was applied to a carbon grid, rinsed with sterile distilled water and stained with 1% (w/v) sodium phosphotungstate, pH 7.0 for 10 s. The excess fluid was removed, the grid air dried and the sample examined using a JEOL 100CX electron microscope at 50,000 × magnification (60 kV). Negatives were printed at a final magnification of 125,000 × .

2.4. Immunization

Eight to 12-week-old female inbred (BALB/c) or outbred (CD1 and CF1) mice were obtained from Charles River (Wilmington, MA). Native and chimeric HBsAg were administered in alum by intraperitoneal injection. Twenty-eight days later blood samples were drawn and analyzed for anti-HBs antibody titers using a radioimmunoassay [24] (AUSAB kit, Abbott Laboratories, Chicago, IL). AUSAB assay was performed according to manufacturer's protocol and samples with less than 2 mIU are considered negative for anti-HBs antibodies [24]. Mice were primed with TT by subcutaneous injection with 50 μg TT in alum (Wyeth Labs, Inc., Marietta, PA) 28 days prior to the initial administration of HBsAg preparations. Booster injections were administered 110 days later. Enhancement of anti-HBS responses was assessed by

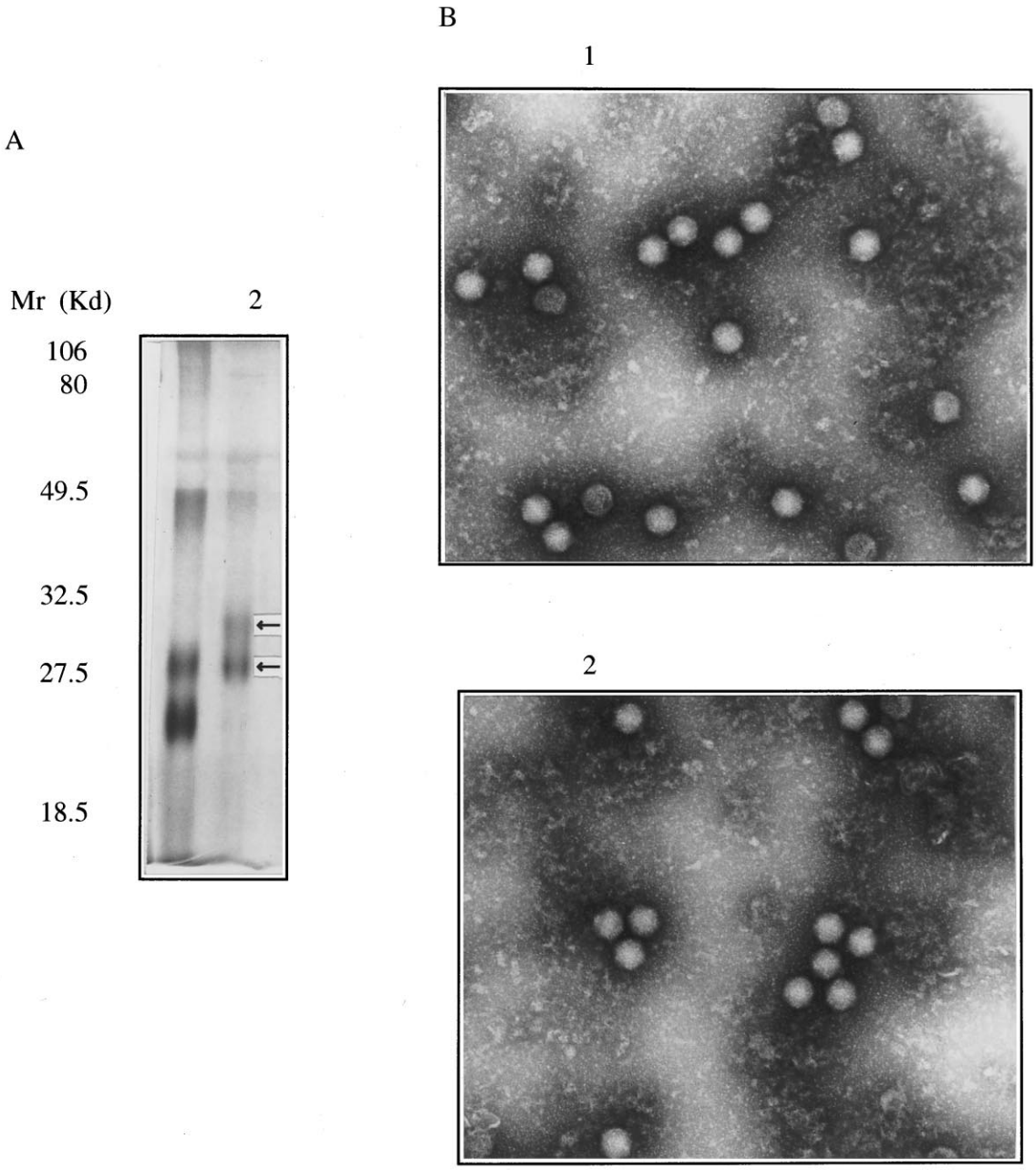

Fig. 1. Characterization of HBsAg carrying TT T_h epitope. HBsAg and HBsAg–TTe produced in an adenovirus expression system were purified by immunoaffinity chromatography. (A) SDS–polyacrylamide gel electrophoretic profile of HBsAg (1) and HBsAg–TTe (2). (B) Electron micrographs of negatively stained HBsAg (1) and HBsAg–TTe (2) at magnification 125,000 × .

Students *t*-test analysis of $\log_{10}$ transformed geometric mean titers.

3. Results

3.1. Characterization of HBsAg–TTe chimeric protein

Inoculation of A549 cells with Ad7ΔE3H-TTe resulted in the secretion of HBsAg–TTe into the culture medium. At 72 h post-infection, culture medium was harvested and the chimeric protein then purified by immunoaffinity chromatography. Examination of purified preparations by electron microscopy indicated that HBsAg–TTe assembled into 22 nm particles that were morphologically similar to particles formed by native HBsAg (Fig. 1). SDS–PAGE analysis of native surface antigen gave two bands, consistent with previous reports of unglycosylated and glycosylated forms of HBsAg [23]. As expected the chimeric protein also showed two bands, each of which showed an increase in molecular weight of about 3 kd due to the TTe

Table 1
Immunogenicity of HBsAg–TTe in BALB/c mice

Immunogen	Dose (ng)	Anti-HBs (mIU) (GMT ± S.E.)[a]	Enhancement[b]
Experiment l:			
HBsAg	100	34.7 ± 16.6	–
HBsAg–TTe	100	523.4 ± 229.2	15.1[c]
HBsAg	12.5	1.4 ± 0.2	—
HBsAg–TTe	12.5	19 ± 12.4	13.6[c]
Experiment 2:			
HBsAg	62.8	3.1 ± 1.3	—
HBsAg–TTe	62.8	430 ± 181	139[c]
HBsAg	7.85	1.4 ± 0.3	—
HBsAg–TTe	7.85	7.1 ± 3.3	5.1[c]

[a]Geometric mean titer (GMT) of all the mice immunized at 28 days post-inoculation ± standard error.
[b]Relative to response following immunization with HBsAg at same dose.
[c]$p < 0.003$, comparison HBsAg vs HBsAg–TTe at same dose.

sequence and appeared to be properly glycosylated (Fig. 1).

3.2. Primary anti-HBs response in inbred and outbred mice

The immunogenicity of HBsAg–TTe was evaluated in BALB/c mice (inbred) at doses ranging from 7.85 to 100 ng per mouse. Anti-HBs antibody responses were measured at 28 days post-immunization (Table 1). HBsAg–TTe consistently induced higher anti-HBs titers than native HBsAg over all doses tested. Increases in the geometric mean titer (GMT) of anti-HBs responses in the HBsAg–TTe-immunized group ranged from 5.1- to 137-fold. All mice immunized with HBsAg–TTe showed significant anti-HBs responses at the 63 ng dose whereas at this dose only 6 out of 15 (40%) mice immunized with native HBsAg showed significant antibody titers.

The immunogenicity of HBsAg–TTe was also evaluated in two outbred mouse strains, CD1 and CF1. In both strains of mice, chimeric protein was significantly more immunogenic than the native protein in eliciting anti-HBs responses after a single dose of 50 ng/mouse; HBsAg–TTe elicited 13.5-fold enhancement in CF1 mice, while the response in CD1 mice was enhanced 60-fold relative to the response induced by native HBsAg (Table 2).

3.3. Effect of priming with TT in mice

Priming with TT has been shown to induce epitopic suppression in various systems where TT is used as a carrier [12–15]. To establish whether priming with TT might have a suppressive effect on subsequent immune responses induced by chimeric HBsAg, BALB/c mice were injected subcutaneously with TT and 28 days later, HBsAg or HBsAg–TTe was administered intraperitoneally. The results presented in Table 3 clearly show a lack of suppression mediated by TT priming. At the two doses tested significantly increased anti-HBs responses (20–54-fold) are seen in the HBsAg–TTe group.

In a follow-up experiment (Table 4), we examined the effect of TT priming on booster responses to HBsAg–TTe administered at low antigen dose (7.5 ng/mouse). BALB/c mice were either initially primed with TT or received no TT priming, whereupon the mice received primary and booster immunizations with either HBsAg or HBsAg–TTe on days 28 and 110, re-

Table 2
Immunogenicity of HBsAg-TTe in outbred mice

Strain	Immunogen	Dose (ng)	Anti-HBs (mIU) (GMT ± S.E.)[a]	Enhancement[b]
CD1	HBsAg	50	2.2 ± 1.0	—
CD1	HBsAg–TTe	50	133 ± 47	60[c]
CF1	HBsAg	50	1.0 ± 0	—
CF1	HBsAg–TTe	50	13.5 ± 7.8	13.5[c]

[a]GMT 28 days post-inoculation ± standard error.
[b]Relative to response following immunization with HBsAg at same dose.
[c]$p < 0.0025$ comparison HBsAg vs HBsAg–TTe in same mouse strain.

Table 3
Immunogenicity of HBsAg–TTe in mice after priming with tetanus toxin: lack of carrier-mediated suppression

Treatment (ng/mouse)		Anti-HBs response (mIU) (GMT ± S.E.)[a]	Enhancement[b]
HBsAg	100	18 ± 12	—
HBsAg–TTe	100	973 ± 581	54[c]
HBsAg	12.5	1.5 ± 0.5	—
HBsAg–TTe	12.5	30 ± 14	20[c]

[a]GMT 28 days post-inoculation ± standard error.
[b]BALB/c mice were injected on day 0 with TT and on day 28 with HBsAg or HBsAg–TTe. Mice were sacrificed 28 days after the last injection and serum anti-HBs titers analyzed.
[c]$p < 0.0001$, comparison HBsAg vs HBsAg–TTe at same dose

spectively. Twenty-eight days after the last injection, serum anti-HBs titers were measured. Although TT priming did not have a significant effect on the primary anti-HBs response, prior treatment with TT resulted in an approximate 6.6-fold increase in booster responses to HBsAg–TTe after the second immunization. The 6.6-fold enhancement in anti-HBs responses is similar to the level of enhancement reported by Etlinger et al. [19] following TT priming in the malarial TTe chimeric polypeptide system.

4. Discussion

The antibody response to HBsAg is the consequence of a complex and highly regulated collaboration involving primarily T_h and B lymphocytes. Following binding of HBsAg to B cell receptors (membrane immunoglobulins), antigen is internalized and processed into peptides (epitopes) which are then presented to T_h cells as complexes with MHC molecules [25–27]. T_h cells play an obligatory role in antibody responses to most protein antigens, providing signals that act on antigen-stimulated B lymphocytes, resulting in their differentiation and clonal expansion into cells that actively secrete antibody [28, 29]. These signals are mediated in part by soluble factors such as IL-2, IL-4, IL-5, interferon gamma, etc [30, 31]. Accordingly, T_h epitopes having the property of promiscuous interaction with a wide range of MHC molecules [32, 33] would be advantageous in stimulating immune responses in the general population. The suboptimal immunogenicity of the currently available HBV vaccines has been attributed alternatively to the presence of weak T_h epitopes in the major HBsAg molecule [25].

In the present study, a fusion gene of HBsAg containing the TTe sequence at amino acid position 50 was constructed and expressed using an adenovirus expression system. It was shown earlier [21] that a foreign epitope inserted in the HBsAg sequence at amino acid position 50 does not significantly alter either the overall conformation or stability of HBsAg particles or binding interactions with anti-HBs antibodies. Secreted HBsAg–TTe particles were found to have morphological characteristics similar to native HBsAg. When tested in an inbred mouse strain (BALB/c), HBsAg–TTe produced stronger antibody responses than similarly prepared HBsAg lacking TTe. The enhanced immunogenicity of HBsAg–TTe was exhibited over a wide dose range. In addition, when evaluated in CD1 and CF1 mice, both outbred strains responded better to HBsAg–TTe than to HBsAg (Table 2), indicating that the TTe epitope was recog-

Table 4
Immunogenicity of HBsAg–TTe in mice after priming with tetanus toxin

Treatment			Anti-HBs response (mIU) (GMT ± SE.)[a]		
1	2	3	Primary	Secondary	Enhancement after secondary immunization
—	HBsAg	HBsAg	1.4 ± 0.3	2.2 ± 1.1	—
TT	HBsAg	HBsAg	9.4 ± 3.7	1.7 ± 0.7	0.78
—	HBsAg–TTe	HBsAg–TTe	7.1 ± 3.3	52 ± 28.8	—
TT	HBsAg–TTe	HBsAg–TTe	5.4 ± 2.2	342 ± 244	6.6[b]

[a]Geometric mean titer (GMT) 28 days post-inoculation ± standard error.
[b]$p = 0.052$, effect of TT priming on immunization with HBsAg–TTe.

nized by the variety of MHC haplotypes present in these outbred strains.

TT is known to contain suppressor epitopes and has been shown in other systems to inhibit anti-hapten antibody responses to TT-hapten in TT-primed hosts [12, 19]. To determine whether TTe exhibits suppressor activity in our system, the effect of priming mice with TT on primary and secondary responses to HBsAg–TTe was studied. No detectable suppressor activity was observed and our results show that TT priming enhanced the subsequent anti-HBs response about 6.6-fold.

These results suggest that the use of TTe should be considered to improve the immunogenicity of hepatitis B vaccines in humans. Improved immunogenicity might result in either a reduction of the amount of antigen used in immunization, a reduction in the number of immunizations required for long term protection or in elevated anti-HBs titers. This approach may have further advantages by eliciting anti-HBs responses in individuals that do not respond well to the current HBsAg vaccines. Finally, because DPT immunization is universal, enhanced anti-HBs responses might result from the use of hepatitis B vaccine containing TTe.

Acknowledgements

The authors wish to thank Mr William Magargle, Ms Barbara Stauffer and Valerie Coleburn for their expert technical assistance and Ms Mary Hason and Phyllis Totaro for typing the manuscript.

References

[1] Szmuness W. Hepatocellular carcinoma and the hepatitis B virus: evidence for a casual association. Prog Med Virol 1978;24:40–69.

[2] Ellis R.W. 1990. New and improved vaccines against hepatitis B. In: New Generation Vaccines. Woodrow G, Levine M, editors. New York: Mercel Dekker Inc. p. 439–447.

[3] Brown SE, Stanley C, Howard CR, Zuckerman AJ, Steward MW. Antibody responses to recombinant and plasma derived hepatitis B vaccines. Br Med J 1986;292:159–61.

[4] Scolnick EM, McLean AA, West DJ, McAleer WJ, Miller WJ, Buynak EB. Clinical valuation in healthy adults of a hepatitis B vaccine made by recombinant DNA. J Am Med Assoc 1984;251:2812–5.

[5] Crosnier J, Jungers P, Courouce AM, Laplanche A, Benhamou E, Degos F, Lacour B, Prunet P, Cerisier Y, Guesry P. Randomized placebo-controlled trial of hepatitis B surface antigen vaccine in French haemodialysis units: II, haemodialysis patients. Lancet 1981;I:797–800.

[6] Lelie PN, Reesink HW, Manen ST, Dees PJ, Brongers EE. Immune response to heat-inactivated hepatitis B vaccine in patients undergoing hemodialysis. Enhancement of the response by increasing the dose of hepatitis B surface antigen from 3 to 27 micrograms. Arch Intern Med 1985;145:305–9.

[7] Degos F, Duhamer G, Brechot C, Nalpas B, Courouce AM, Tron F, Berthelot P. Hepatitis B vaccination in chronic alcoholics. J Hepatol 1986;2:402–9.

[8] Menderhall C, Roselle GA, Lybecker LA, Marshall LE, Grossman CJ, Myre SA. Hepatitis B vaccination. Response of alcoholics with and without liver injury. Dig Dis Sc 1988;33:263–9.

[9] Hess G, Rossol S, Voth R, Cheatham-Speth D, Clemens R, Meyer Zum Buschenfelde KM. Active immunization of homosexual men using a recombinant hepatitis B vaccine. J Med Virol 1989;29:229–31.

[10] Hauser P, Voet P, Simoen E, Thomas HC, Petre J, De Wilde M, Stephenne J. Immunological properties of recombinant HBsAg produced in yeast. Postgraduate Med J 1987;83(91):.

[11] Pillot J, Poynard T, Elias A, Maillard J, Lazizi Y, Brancer M, Dubreuil P, Budkowska A, Chaput JC. Weak immunogenicity of the preS2 sequence and lack of circumventing effect on the unresponsiveness to the hepatitis B virus vaccine. Vaccine 19?;13:289–94.

[12] Schutze MP, Leclerc C, Jolivet M, Audibert F, Chedid LJ. Carrier induced epitopic suppression, a major issue for future synthetic vaccines. J Immunol 1985;135:2319–22.

[13] Herzenberg LA, Tokuhisa T, Herzenberg LA. Carrier priming leads to hapten-specific suppression. Nature 1980;285:664–7.

[14] Herzenberg LA, Tokuhisa T. Epitope-specific induction of suppression for IgG anti-hapten antibody response. J Exp Med 1982;155:1730–40.

[15] Schutze MP, Leclerc C, Vogel FR, Chedid L. Epitopic suppression in synthetic vaccine models: analysis of the effector mechanisms. Cell Immunol 1987;104:79–90.

[16] Tagawa M, Tokuhisa T, Ono K, Taniguchi M, Herzenberg LA, Herzenberg LA. Epitope specific regulation. IV. In Vitro studies with suppressor T cells induced by carrier/hapten-carrier immunization. Cell Immunol 1984;86:327–36.

[17] Sercarz EE, Yowell RL, Turkin D, Miller A, Araneo BA, Adorini L. Different functional specificity repertoires for suppressor and helper T cells. Immunol Rev 1978;39:108–36.

[18] Adorini L, Harvey MA, Miller A, Sercarz EE. Fine specificity of regulatory T cells. II. Suppressor and helper T cells are induced by different regions of hen egg-white lysozyme in a genetically nonresponder mouse strain. J Exp Med 1979;150:293–306.

[19] Etlinger HM, Gillssen D, Lahn HW, Matile H, Schonfeld HJ, Trzeciak A. Use of prior vaccinations for the development of new vaccines. Science (USA) 1990;249:423–5.

[20] Delpeyronx F, Van Wezel E, Blondel B, Carinic R. Structural factors modulate the activity of antigenic poliovirus sequences expressed on hybrid hepatitis B surface antigen particles. J Virol 1990;64:6090–100.

[21] Chengalvala MV, Bhat BM, Bhat RA, Lubeck MD, Mizutani S, Davis A, Hung PP. Immunogenicity of high expression adenovirus-hepatitis B virus recombinant vaccines in dogs. J Gen Virol 1994;75:125–31.

[22] Chengalvala MV, Bhat BM, Bhat RA, Dheer SK, Lubeck MD, Purcell RH, Murthy KK. Replication and immunogenicity of Ad7-, Ad4-, and Ad5-hepatitis B virus surface antigen recombinants, with or without a portion of E3 region, in chimpanzees. Vaccine 1997;15:335–9.

[23] Molnar-Kimber KL, Jarocki-Witek V, Dheer SK, Vernon SK, Conley AJ, Davis AR, Hung PP. Distinctive properties of the hepatitis B virus envelope proteins. J Virol 1988;62:407–16.

[24] Maupas P, Coursaget P, Goudeau A, Drucker J, Bagros P. Immunization against hepatitis B in man. Lancet 1976;1:1367–70.

[25] Milich DR. Synthetic T and B cell recognition sites: implications for vaccine development. Advances in Immunology 1989;45:195–264.

[26] Milich DR. T- and B-cell recognition of hepatitis B viral antigens. Immunology Today 1988;9:380–6.
[27] Berzofsky JA. Ir Genes: antigen-specific genetic regulation of the immune response. The Antigens 1987;7:1–113.
[28] Owens T. A role for adhesion molecules in contact dependent T help for B cells. Eur J Immunol 1991;21:979–83.
[29] Lamb JR, Ivanyi J, Rees ADM, Rothbard JB, Howland K, Young RA, Young DB. Mapping of T cell epitopes using recombinant antigens and synthetic peptides. EMBO Journal 1987;6:1245–9.
[30] Perlmutter RM. T cell signaling. Science (USA) 1989;245:344–6.
[31] Cardell S, Sander B, Moller G. Helper interleukins are produced by both CD4 and CD8 splenic T cells after mitogen stimulation. Eur J Immunol 1991;21:2495–500.
[32] Sinigaglia F, Guttinger M, Kilgus J, Doran DM, Matile H, Etlinger H, Trzeciak A, Gillessen O, Pink JRL. A malaria T cell epitope recognized in association with most mouse and human class II molecules. Nature 1988;336:778–80.
[33] Partidos CD, Steward MW. Prediction and identification of a T cell epitope in the fusion protein of measles virus immunodominant in mice and humans. J Gen Virol 1990;71:2099–105.