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Published

*With international search report.*

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**Title:** EPITOPES OF THE PRE-S REGION OF HEPATITIS B VIRUS SURFACE ANTIGEN

**Abstract**

Polypeptides corresponding in amino acid residue sequence to native T cell epitopes in the pre-S (2) region of HBsAg are disclosed. A method of mitigating nonresponsiveness to HBsAg comprising including T cell epitopes of both the d and the y subtypes is also disclosed.
| AT  | Austria       | ES  | Spain       | MG  | Madagascar  |
| AU  | Australia     | FI  | Finland     | ML  | Mali        |
| BB  | Barbados      | FR  | France      | MN  | Mongolia    |
| BE  | Belgium       | GA  | Gabon       | MR  | Mauritania  |
| BF  | Burkina Faso  | GB  | United Kingdom | MW  | Malawi     |
| BG  | Bulgaria      | GN  | Guinea       | NL  | Netherlands |
| BJ  | Benin         | GR  | Greece       | NO  | Norway       |
| BR  | Brazil        | HU  | Hungary      | PL  | Poland       |
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| CF  | Central African Republic | JP  | Japan       | SD  | Sudan        |
| CG  | Congo         | KP  | Democratic People's Republic | SE  | Sweden   |
| CH  | Switzerland   | KR  | Republic of Korea | SN  | Senegal |
| CI  | Côte d'Ivoire | LI  | Liechtenstein | SU  | Soviet Union |
| CM  | Cameroon      | LK  | Sri Lanka    | TD  | Chad         |
| CS  | Czechoslovakia | LU  | Luxembourg   | TG  | Togo         |
| DE  | Germany       | MC  | Monaco       | US  | United States of America |
EPITOPES OF THE PRE-S REGION OF
HEPATITIS B VIRUS SURFACE ANTIGEN

Description

Cross Reference to Related Application

This application is a Continuation-In-Part of application Serial No. 060,214 filed June 10, 1987, which application is a Continuation-In-Part of application Serial No. 877,020, filed June 20, 1986, the disclosures of which are incorporated herein by reference.

Technical Field Of The Invention

The present invention relates to the production of novel polypeptides related to the hepatitis B virus surface antigen (HBsAg) protein and to the use of those polypeptides in vaccines, diagnostic reagents, and the like.

Background Of The Invention

Viral hepatitis continues to rank as one of the most important unconquered diseases of mankind. The general term, viral hepatitis, refers principally to hepatitis A (infectious hepatitis), to hepatitis B (serum hepatitis) and to non-A, non-B hepatitis, although other known viruses such as yellow fever virus, Epstein-Barr virus and cytomegalovirus can cause hepatitis in man. Hepatitis is particularly known for its focal attack on the liver (Greek, hepâr), but the disease also influences other organs.

It would be desirable to produce a vaccine and resultant antibodies that would provide protection against Hepatitis B virus (HBV) and the diseases it causes. Historically, vaccines and antibodies have
been prepared by killing or attenuating viruses and then injecting the resulting virus particles into a patient or host animal. However, such vaccines always have the inherent threat that the virus may not be completely killed or sufficiently attenuated. The "vaccine" sometimes itself causes disease.

The threat of unattenuated viruses can sometimes be overcome by using only a portion of the virus. This portion is usually a protein from a capsid or envelope which forms the outer portion of the virus. However, even this method is not without well-known difficulties including possible pathogenic responses. The produced vaccine may include antigens that compete with or are even detrimental to the desired immune response. Other antigenic material may also be present that is unrelated to the desired immune response and can cause undesirable side effects.

Furthermore, HBV does not multiply efficiently in cell culture, consequently, there is no available tissue culture source for HBV as immunogen.

Various attempts have been made to manufacture vaccines and antibodies to other diseases by other methods. These methods include producing antigen and antibody-producing cells by recombinant DNA techniques and hybridoma methods. However, these methods in addition to being relatively complicated and expensive, are time consuming and have relatively low yields both quantitatively and qualitatively. Great care must be taken in preparing the vaccine or antibody producing cell and in harvesting the desired product. There is also concern about the safety and reliability of any method that requires the desired product to be separated from undesired, possibly pathogenic components.
In 1965, Blumberg discovered an antigen circulating in the blood of certain human beings; (1965) J. Am. Med. Assoc., 191:541 and (1967) Ann. Int. Med., 66:924. This substance was subsequently found by Prince to be the surface antigen of hepatitis B virus (HBsAg) that is produced in abundance by individuals who are chronically infected with the viral agent; (1968) Proc. Natl. Acad. Sci. (USA), 60:814.


The large open reading frame (ORF) for HBsAg terminates in a single stop codon but can initiate at three possible translational start codons, that define the pre-S1, pre-S2, and S regions, yielding polypeptides referred to as p39, p33 and p25, respectively. All three polypeptides share the 226 amino acid residues of the S region (p25). The p33 consists of the p25 sequence plus an amino-terminal 55 residues (pre-S2); and p39 consists of the p33 sequence plus an amino-terminal 119 residues (pre-S1) in the adw subtype or 108 residues in the ayw subtype due to a NH2-terminal deletion of 11 residues;

Tiollais et al, (1981) Science 213:406. Amino acid residue positions of the pre-S region of HBsAg are numbered to the carboxy terminus and such that position 120 is the first position in the pre-S(2) region polypeptide of all subtypes of HBV. The numbering of positions begins again in the S region.
polypeptide with position 1 designated as the amino-terminal residue.


Although there is an urgent need for a hepatitis B vaccine for groups that are at an increased risk of acquiring this infection, as stated above, hepatitis A and B viruses, do not multiply significantly in cell culture; consequently, there is no current source of laboratory-propagated virus for vaccine preparation. Vaccines for HBV consist of subviral components of the virus surface coat (HBsAg) purified from the plasma of chronically HBV-infected donors and inactivated; McAuliffe, et al., (1980) Rev. Infect. Dis., 2:470. The purification process produced particles containing HBsAg/p25 but not HBsAg/p33 or HBsAg/p39. Clinical trials have demonstrated the safety and efficacy of current HBsAg vaccines but a significant percentage of people are not protected by inoculation with such vaccines due to a genetic inability to respond to HBsAg/p25 (S region nonresponders).
In mice, the in vivo antibody production to HBsAg is regulated by at least two immune response (Ir) genes, one in the I-A subregion (Ir-HBs-1) and one in the I-C subregion (Ir-HBs-2) of the murine H-2 complex.

The linkage between the major histocompatibility complex and the regulation of immune responsiveness to HBsAg in mice has been extended to the human immune response by the report of an association between HLA-DR phenotype and nonresponsiveness to a recent trial HBsAg vaccine.

In response to the finding that the pre-S region contains T cell epitopes that induce responsiveness in some S-region nonresponding mouse strains, recent vaccine formulations have included a portion of the pre-S region. In particular, one commercially available vaccine contains HBsAg/p33\text{ay} and another contains HBsAg/p33\text{ad}.

Thus, the construction of an HBV vaccine ideally should include, in addition to B cell immunogenic epitopes, a sufficient diversity of T cell epitopic determinants to accommodate the genetic variation in epitope recognition of an outbred human population.

Summary Of The Invention

T cell epitopes of the HBsAg pre-S(2) region have now been determined. In particular, specific sequences of the pre-S(2) region, located between residues 148 and 174, have now been shown to function as T cell epitopes. Composite polypeptides comprising the T cell epitopes operatively linked to the an HBsAg B cell epitope, usually a native group-specific epitope, in specific orientations, have been determined to be effective immunogens.
In determining specific T cell epitope sequences, it has been found that animals having different H-2 haplotypes recognize different portions of the pre-S(2) region as T cell epitopes. Further, even within one animal strain, different regions of the \( \delta \) and \( \gamma \) subtypes may be recognized as T cell epitopes. An animal may also recognize a T cell epitope in the pre-S(2) region of one subtype but not the other. Thus, the present invention contemplates the use of T cell epitopes of both subtypes to prime or vaccinate to induce responsiveness to an HBV vaccine.

One aspect contemplated by the present invention is a pre-S(2) T cell epitope polypeptide of 6 to 50 amino acid residues consisting essentially of an amino acid residue sequence that corresponds to at least one amino acid residue sequence selected from the group consisting of:

residues 136-155, 136-174, 148-159, 148-174, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/\( \delta \); and residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/\( \gamma \).

This polypeptide is free of amino acid residues at the amino-terminal and the carboxy-terminal positions that correspond to contiguous amino acid residues in the linear sequence of HBsAg.

Another aspect contemplated is a composite polypeptide comprising a first polypeptide (a) operatively linked to the amino terminus of a second
polypeptide (b). Polypeptide (a) consists essentially of a pre-S(2) T cell epitope polypeptide of 6 to 50 amino acid residues including an amino acid residue sequence that corresponds to at least one amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 148-159, 148-174, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/d and residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/α. Polypeptide (b) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence of HBsAg that contains a native B cell epitope.

In preferred embodiments, the composite polypeptide B cell epitope is a group-specific B cell epitope, which may contain an amino acid residue sequence that corresponds to subtype-specific B cell epitopes, an amino acid residue sequence that corresponds to an S region B cell epitope, an amino acid residue sequence that corresponds to a pre-S(1) region B cell epitope or a pre-S(2) region B cell epitope or a pre-S(2) T cell epitope polypeptide that corresponds to residues 148-174 of the pre-S region of HBsAg/d or HBsAg/α.

Another aspect contemplated by this invention is a composite polypeptide of at least 14 and not more than 100 amino acid residues comprising a first polypeptide (a) linked to the amino terminus of a second polypeptide (b) wherein polypeptide (a) comprises a pre-S(2) T cell epitope having an amino acid residue sequence that corresponds to at least one amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 148-159,
148-174, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/d and residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/y. Polypeptide (b) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence of HBsAg that contains a native B cell epitope.

In preferred embodiments, this polypeptide (b) is a group-specific epitope, which may possess an amino acid residue sequence that corresponds to a native pre-S(2) region B cell epitope, an amino acid residue sequence that corresponds to an amino acid residue sequence of the pre-S(2) region of HBsAg selected from the group consisting of residues 133-139, 133-143, and 137-143, and an amino acid residue sequence corresponding to at least one amino acid residue sequence selected from the group consisting of residues (141-174)-(133-140), (146-160)-(133-143), (148-174)-(133-143), (151-165)-(133-143), (151-174)-(133-143) of the pre-S(2) region of HBsAg.

Also contemplated is a composite subtype T cell hepatitis B virus (HBV) vaccine comprising polypeptides (a1) and (a2) wherein polypeptide (a1) comprises an amino acid sequence corresponding to the amino acid residue sequence of a native HBsAg/d T cell epitope and polypeptide (a2) comprises an amino acid residue sequence corresponding to the amino acid residue sequence of a native HBsAg/y T cell epitope.

Preferred embodiments may contain a native pre-S region T cell epitope, (a1) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence selected from the group
consisting of residues 136-155, 136-174, 148-159, 148-174, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/d, (a2) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/y, and additionally comprising at least one of polypeptide (b1) and polypeptide (b2) which is operatively linked to at least one of polypeptide (a1) and polypeptide (a2), wherein polypeptides (b1) and (b2) each comprise an amino acid residue sequence corresponding to the amino acid residue sequence of a native HBsAg native B cell epitope.

Other embodiments contain polypeptides (a1) and (a2) are operatively linked to polypeptides (b1) and (b2), respectively, and may have the same amino acid sequence, or be operatively linked to polypeptide (b1).

In still other embodiments, the vaccine has at least one of said polypeptides (a1) and (a2) is present in a particle, and may have both polypeptides form a heterogeneous particle.

Also contemplated by this invention is a method of inducing responsiveness to a HBV vaccine comprising administering, either prior to or together with administration of the vaccine, an effective amount of a composite subtype T cell immunogen with at least one native HBsAg/d pre-S T cell epitope and at least one native HBsAg/y pre-S T cell epitope.

In one embodiment of this method, the immunogen can comprise an amino acid residue sequence that corresponds to an amino acid residue sequence
selected from the group consisting of residues 136-155, 136-174, 148-159, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/d and an amino acid residue sequence that corresponds to an amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/y.

In one embodiment, the immunogen and the vaccine are administered simultaneously as a composition that comprises HBsAg/p39ad and HBsAg/p39ay.

Also contemplated by this invention is a synthetic carrier moiety comprising the HBV pre-S(2) sequence 148-174 operatively linked to one or more polypeptide immunogen. In a preferred embodiment, the carrier moiety is linked to the N-terminal of the polypeptide immunogen.

Brief Description Of The Drawings

Figure 1 illustrates the amino acid sequence of the pre-S region of six subtypes of HBsAg.

Figure 2 illustrates the T cell proliferative response of B10.S (circles) and B10.M (squares) mice which were primed with HBsAg/p39ad (open circles, open squares) or HBsAg/p39ay (closed circles, closed squares) to subsequent challenge with HBsAg/p33ad.

The X axis represents the amount of HBsAg/p33ad used for T cell proliferation study in µg/ml. The Y axis represents the amount of $^3$H-thymidine (TdR[$^3$H]) incorporated into the T cells in counts per minute (CPM×10^{-3}).
Figure 3 in FIGS. 3A and 3B illustrates that the B10.S, pre-S(2)-specific T cell proliferative response is subtype-specific. A group of 5 B10.S mice was immunized with 4.0µg of (A) HBsAg/P33d or (B) HBsAg/P33y, and 10 days later draining popliteal lymph node (PLN) cells were analyzed for in vitro T cell proliferation. The antigens tested for induction of T cell proliferation included pre-S(2)-containing HBsAg/P33 particles of the d and y subtypes and HBsAg/P25 particles of both subtypes, which lack the pre-S(2) region. T cell proliferation is expressed as [³H]-TdR uptake corrected for background proliferation in the absence of antigen (CPM). Background proliferation ranged from 850-1500 cpm.

Figure 4 in FIGS. 4A and 4B shows that the B10.M, pre-S(2)-specific T cell proliferative response is subtype-specific. A group of 5 B10.M mice was immunized with 4.0 µg of (A) HBsAg/P33d or (B) HBsAg/P33y, and 10 days later draining PLN cells were analyzed for in vitro T cell proliferation elicited by the indicated panel of antigens as described in Figure 3.

Figure 5 demonstrates that the truncated pre-S(2) polypeptide (P28) can substitute for the full length pre-S(2) polypeptide (P33) in terms of T cell recognition. Groups of 4 mice each of the indicated strains were immunized with 4.0 µg of HBsAg/P33d, and the in vitro T cell proliferative responses elicited by varying concentrations of HBsAg/P33d (P33) or HBsAg/P28d (P28) were determined. The responses shown represent in vitro antigen concentrations of 1.0 µg/ml.

Figure 6 in FIGS. 6A and 6B shows the fine specificity and subtype dependence of B10 strain [T cell recognition of the p133-174 sequence of the pre-S(2) region]. Groups of 5 B10 (H-2b) mice were immunized with 100 µg
of synthetic peptide representing (A) residues 133-174 of the d subtype or (B) residues 133-174 of the y subtype of the pre-S(2) region. Ten days later PLN cells were collected and analyzed for T cell activation elicited by HBsAg/P33 particles of the appropriate subtype or a panel of synthetic peptides derived from the C-terminal pre-S(2) sequence. The subtype of each sequence is indicated.

Figure 7 in FIGS. 7A and 7B shows the fine specificity and subtype-dependence of B10.M strain [T cell recognition of the p133-174 sequence of the pre-S(2) region]. Groups of 5 B10.M (H-2^d) mice were immunized with 100 µg of (A) p133-174d or (B) p133-174y, and T cell proliferation induced by the indicated panel of antigens was determined as described in Figure 6.

Figure 8 in FIGS. 8A and 8B shows the fine specificity and subtype-dependence of B10.S strain [T cell recognition of the p133-174 sequence of the pre-S(2) region]. Groups of 5 B10.S (H-2s) mice were immunized with 100 µg of (A) p133-174d or (B) p133-174y, and T cell proliferation induced by the indicated panel of antigens was determined as described in Figure 6.

Figure 9 in FIGS. 9A and 9B shows the fine specificity and subtype-dependence of B10.D_2 strain [T cell recognition of the p133-174 sequence of the pre-S(2) region]. Groups of 5 B10.D_2 (H-2d) mice were immunized with 100 µg of (A) p133-174d or (B) p133-174y, and T cell proliferation induced by the indicated panel of antigen was determined as described in Figure 3.

Figure 10 depicts a summary of T cell recognition sites within the d and y subtypes of the pre-S(2) region identified amongst a panel of murine strains representing 8 distinct H-2 haplotypes. The amino acid sequence of the p148-174 region of the adw_2
subtype is shown, and the amino acid sequence of the ayw subtype is indicated by boxed letters. Similarly, T cell recognition sites relevant to the adw₂ subtype sequence are indicated by dashed lines. The sites depicted do not necessarily represent the minimum size required to induce T cell proliferation, but represent sequences capable of inducing T cell proliferation at least 10-fold greater than background at a concentration of 10 µg/ml. The immunogens used to prime the T cells used to define these sites included: HBsAg/P33 d and y, p133-174 d and y, and p148-174d. N.D., not determined.

Figure 11 in FIGS. 11A and 11B shows that priming with the native HBsAg/P33 elicits T cells reactive with peptide antigens derived from the C-terminal half of the pre-S(2) region. Groups of 4 B10(A) or B10.M(B) mice were immunized with 4.0 µg of HBsAg/P33d, and PLN cell T cell proliferative responses specific for the indicated panel of antigens determined as described in Figure 3.

Figure 12 demonstrates how the context of a T cell site can influence its immunogenicity. Groups of four B10.S mice were immunized with 100 µg of the indicated peptide immunogens in complete Freund’s adjuvant (CFA). Ten days later draining PLN cells were harvested and T cell proliferative responses induced by a 20 µg/ml concentrations of p156-170 (open bar) and p161-174 (solid bar) were analyzed. The data is expressed as a percent of the proliferation induced by the immunizing peptide, corrected for background.

Figure 13 is a schematic representation of the T and B cell responses to native and synthetic pre-S(2) immunogens. B₁ and B₂ represent dominant antibody binding sites, and T₁ and T₂ represent dominant T cell recognition sites in the B10.S strain.
The presence of an encircled symbol denotes T cell recognition of the indicated T cell site. Antibody production is indicated by lines radiating from the B cell site.

Detailed Description Of The Invention

I. Introduction

It has been found that inoculation of some strains of animals that do not respond to the pre-S(2) and S regions of HBsAg (hepatitis B virus surface antigen) with immunogens containing pre-S(1) region T cell epitopes stimulated the production of antibody to both the pre-S(2) and S regions in addition to pre-S(1) specific antibody, thereby bypassing nonresponsiveness to a hepatitis B virus (HBV) vaccine (Milich, et al, J. Immunol., 137:315, 1986). To facilitate construction of wholly or partially synthetic vaccines, amino acid sequences which function as T cell epitopes have been investigated. In one aspect of this invention, T cell epitopes of HBsAg pre-S(2) region for use to prime or vaccinate a host animal are contemplated. Those T cell epitopes have now been determined to be located between residues 136 and 174 with the majority of T cell epitopes located between residues 148 and 174 of the pre-S(2) region.

Additionally, different portions of the pre-S(2) region have now been shown to function as T cell epitopes in different strains of the same species of animal. Further, within one strain, different regions of the d and y subtypes may be recognized as T cell epitopes or a strain may recognize a T cell epitope in the pre-S(2) region of one subtype but not recognize any T cell epitope in the pre-S(2) region of the other subtype. Thus, in another aspect, the present
invention contemplates the use of T cell epitopes of both subtypes to prime or vaccinate a host animal to mitigate nonresponsiveness to an HBV vaccine.

The amino acid residue sequence of the pre-S region of six subtypes of HBV are illustrated in Figure 1. The single letter amino acid code used in the figure is illustrated below in Table 1.

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<th>Amino Acid</th>
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<td>Alanine</td>
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<td>Asparagine</td>
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<td>N</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Asparagine or aspartic acid</td>
<td>Asx</td>
<td>B</td>
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<tr>
<td>Cysteine</td>
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<td>C</td>
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<td>Glutamine</td>
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<td>Glutamic acid</td>
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II. Polypeptides
A. Pre-S(2) T Cell Epitope Polypeptides

The present invention provides HBsAg pre-S(2) T cell epitope polypeptides. The pre-S(2) T cell epitope polypeptides of this invention are native HBsAg T cell epitopes. That is, the polypeptides of this invention prime T cell help relevant to native HBsAg in addition to T cell help relevant to the polypeptide. Thus, when the pre-S(2) T cell epitope polypeptides of this invention are used to prime a host animal, the primed T cells proliferate in response to HBsAg and provide T cell help in B cell recognition of HBsAg.

A T cell epitope polypeptide of this invention includes about 6 to about 50 amino acid residues comprised of an amino acid residue sequence that corresponds to at least one amino acid residue sequence selected from the group consisting of:

residues 136-155, 136-174,
148-159, 148-174, 149-165, 152-159,
154-170, 156-165, 156-167, 156-170,
159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/Δ; or
151-165, 151-170, 156-165, 159-169,
and 161-174 of the pre-S(2) region of HBsAg/Y.

For convenience, the abbreviation p___-____ (or ___-___) will be used herein to refer to a polypeptide corresponding to a portion of the pre-S region, where the blanks indicate the amino- and carboxy-terminal positions of the peptide, respectively. The T cell epitope polypeptide can include not more than 10, preferably not more than 5, amino acid residues at each of the amino-terminal and the carboxy-terminal
positions of the recited sequences that correspond to contiguous amino acid residues in the linear sequence of HBsAg. Most preferably, the T cell epitope polypeptide is free of amino acid residues at both the amino-terminal and the carboxy-terminal positions that correspond to contiguous amino acid residues in the linear sequence of HBsAg. A T cell epitope polypeptide of this invention may include a plurality of the above-described sequences or repeats or partial repeats of the sequences.

Native T cell epitopes for various mouse strains are indicated in Table 2 below. The procedure for determining the relevant T cell epitopes is described in detail in the examples.

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<tr>
<td>B10.P</td>
<td>149-165</td>
<td>151-170</td>
</tr>
<tr>
<td>B10</td>
<td>148-159</td>
<td>136-155</td>
</tr>
<tr>
<td></td>
<td>152-159</td>
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<td>148-155</td>
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<td>148-165</td>
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<tr>
<td>B10.BR</td>
<td>156-165</td>
<td>156-165</td>
</tr>
<tr>
<td>B10.M</td>
<td>156-167</td>
<td>N.R.</td>
</tr>
<tr>
<td>B10.D2</td>
<td>156-170</td>
<td>146-165</td>
</tr>
<tr>
<td>B10.S</td>
<td>154-170</td>
<td>161-174</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>159-169</td>
<td>159-169</td>
</tr>
<tr>
<td>B10.RIII</td>
<td>159-167</td>
<td></td>
</tr>
</tbody>
</table>

The amino acid residues that comprise the above sequences are listed in Figure 1. As shown in that figure, there are sequences illustrated for six HBsAg subtypes, and that all of the amino acid
residues are not the same at a given position. As used herein, HBsAg sequences are intended to include the z'igned sequences in both the glycosylated and nonglycosylated forms for all HBV subtypes unless otherwise stated.

Amino acid residues present in a T cell epitope polypeptide of the invention, in addition to a sequence corresponding to a sequence described hereinbefore, can be any residues that do not materially affect the basic and novel characteristics of a polypeptide as are discussed hereinafter. Such additional residues are usually added to one or both termini of a described polypeptide and can include repeats and partial repeats of a polypeptide sequence or contiguous residues of the HBsAg protein sequence.

One or more residues may be added, usually at the carboxy terminus, for the purpose of providing a "linker" by which the polypeptides of this invention can be conveniently operatively linked together or, usually, to an HBsAg B cell epitope polypeptide to form a composite polypeptide of this invention, as described in detail hereinafter. Amino acid residue linkers are usually at least one residue and can be 40 or more residues, more often 1 to 10 residues.

Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic and aspartic acid, or the like. In addition, a polypeptide sequence of this invention can differ from the natural sequence by the sequence being modified by terminal-NH₂ acylation, e.g., acetylation, carboxylamidation, e.g., ammonia, methylamine, etc.

A T cell epitope polypeptide sequence of the present invention has an amino acid residue sequence that corresponds to a portion of the amino acid residues sequence of HBsAg. Thus, a T cell epitope
polypeptide of the present invention need not be
identical to the amino acid residue sequence of HBsAg.
Usually the peptide analogues will retain
substantially identical T cell proliferation activity.
Therefore, a T cell epitope polypeptide can be subject
to various changes, such as insertions, deletions and
substitutions, either conservative or non-
conservative, where such changes provide for certain
advantages in their use and maintain the important
functional attribute of T cell stimulation.

Conservative substitutions are those where
one amino acid residue is replaced by another,
biologically similar residue. Examples of
conservative substitutions include the substitution of
one hydrophobic residue such as isoleucine, valine,
leucine or methionine for another, or the substitution
of one polar residue for another such as between
arginine and lysine, between glutamic and aspartic
acids or between glutamine and asparagine and the
like. The term "conservative substitution" also
includes the use of a substituted amino acid in place
of an unsubstituted parent amino acid provided that
such a polypeptide also displays the requisite binding
activity.

When a polypeptide of the present invention
has a sequence that is not identical to a portion of
the sequence of HBsAg because one or more deletions,
insertions or conservative or non-conservative
substitutions have been made, usually not more than
about 20% and more frequently not more than 10% of the
amino acid residues differ. As used herein, sequences
which "substantially correspond" to a specified
sequence are those having not more than 10% deletions,
insertions or substitutions. Of course additional
residues may be added at either terminus. The
additional residues may correspond to contiguous residues in the linear sequence of HBsAg as described previously, or may be repeats of the sequence or amino acid residues that are unrelated to HBsAg.


A T cell epitope polypeptide finds use in priming an animal to stimulate T cell recognition of HBsAg either prior to or together with vaccine administration. A T cell epitope polypeptide of this invention also finds use as an immunogen that primes T cells that respond to native HBsAg when the T cell epitope is attached to an HBsAg B cell epitope polypeptide.

A T cell epitope polypeptide of this invention is also useful as a substitute for well known immunogens such as KLH, tetanus toxoid and bovine gamma globulin when a specific induction of antibody is desired. The T cell epitope peptides of this invention are safe, defined and T cell-active, making them advantageous substitutes for heterologous immunogens.

Generally, the T cell epitope polypeptides of this invention find use to prime T cells that respond to native HBsAg of the same subtype. However, when attached to HBsAg B cell epitope polypeptides, these
peptides stimulate the production of antibody to HBsAg in addition to priming T cells.

B. B cell Epitope Polypeptides

A B cell epitope may be subtype-specific or a group-specific (also referred to as a cross-reactive epitope), most frequently group-specific. A B cell epitope polypeptide comprises an amino acid sequence of at least six amino acid residues and may include several hundred residues. This epitope can comprise an amino acid residue sequence that corresponds to a native B cell epitope that is free from contiguous residues in the HBsAg sequence that are not involved in the antibody recognition site, or alternatively, it can be either natural HBsAg, or the S, pre-S(1) or pre-S(2) regions or fragments or combinations thereof.


III. Composite Polypeptides

A. Description

A composite polypeptide of the present invention comprises a first polypeptide (a) which is operatively linked to a second polypeptide (b). Polypeptide (a) comprises a pre-S(2) T cell epitope polypeptide of this invention. Polypeptide (b) comprises an amino acid residue sequence that corresponds to a native HBsAg B cell epitope. By
"operatively linked" it is meant that the T cell epitope polypeptide is covalently bound to the B cell epitope polypeptide. The covalent bond can be a peptide bond so that the peptidic [polypeptides (a) and (b)] of the composite are linked to form a single primary structure. Alternatively, the peptidic submits corresponding to polypeptides (a) and (b) can be linked by other than a peptide bond, e.g. through an amino acid residue side chain, thereby forming what is typically known in the art as a "conjugate". The composite polypeptides can have one or more of the same or different pre-S(2) T cell epitope polypeptide linked to one or more B cell epitope polypeptide. In one embodiment, the composite polypeptide comprises one or more pre-S(2) T cell epitope polypeptide linked to a relatively large fragment of HBsAg, such as p25, p33 or p39. When the fragment includes the pre-S(2) region, the T cell epitope will be of a different subtype than the B cell epitope (e.g., recombinant p33/d + 148-174/y). However, when the pre-S(2) region is not present in the fragment, i.e., when the fragment is HBsAg/p25, the T cell epitope may be either or both subtypes. Thus, either one or a plurality of pre-S(2) T cell epitopes can be included in a composite polypeptide.

In a preferred embodiment, the composite polypeptides are relatively short amino acid residue sequences and are, therefore, easily synthesized chemically. In that embodiment, the composite polypeptides include at least about 14, and not more than about 100 amino acid residues; preferably not more than about 50 amino acid residues; and more preferably not more than about 30 amino acid residues. In that same embodiment, a single pre-S(2) T cell epitope polypeptide will usually be linked to a single
B cell epitope polypeptide. The pre-S(2) T cell epitope may be of the same or different subtype from the B cell epitope.

Polypeptides (a) and (b) can be separated by a spacing group. Such spacing groups are preferably short polymers of one to about ten amino acid residues. Usually, those residues are not contiguous residues in the HBsAg linear sequence, but are selected from another part of the HBsAg molecule or an unrelated sequence. Nonsense sequences such as polyglycine can also be used.

In a more preferred embodiment, which yields unexpectedly good results, the pre-S(2) T cell epitope of the composite polypeptide is linked directly to the N-terminus of a HBsAg B cell epitope polypeptide. Pre-S(2) B cell epitope polypeptides useful in these composite arrangements include residues 133-139, 133-140, 133-143, and 137-143. These more preferred pre-S(2) composite polypeptides include the following sequences: (p141-174)-(p133-140), (p146-160)-(p133-143), (p148-174)-(p133-143), (p151-165)-(p133-143) and (p151-174)-(p133-143) of the pre-S(2) region of HBsAg.

Although antibody may be elicited by binding a pre-S(2) T cell epitope polypeptide to the C-terminus of the B cell epitope polypeptide, the orientation in which the peptides are linked has now been found to be important in eliciting antibody directed to the desired B cell epitope, at least for pre-S(2) B cell epitopes. Studies describing this principle are recounted in the examples.

To determine the optimal orientation for short composite polypeptides having a pre-S(1) or S B cell epitope, similar studies can be performed.

B. Synthesis
When the composite polypeptide has a relatively short amino acid residue sequence, it can be synthesized by chemical techniques as described for the pre-S(2) T cell epitope polypeptides hereinbefore. However, when the composite polypeptide is relatively long, typically, the T cell epitope peptide is chemically synthesized and subsequently linked to the B cell epitope polypeptide. The B cell epitope polypeptide can be chemically synthesized, produced by recombinant techniques or isolated from plasma.

Gene segments for the S, pre-S(1) and pre-S(2) regions of HBsAg are known, and can be appropriately linked together and expressed from microorganisms such as bacteria like E. coli or yeast like S. cerevisiae, as well as by mammalian cells such as Chinese hamster ovary (CHO) cells to provide B cell epitope polypeptides. For example, Tiollais and co-workers [Michel, et al, Proc. Natl. Acad. Sci. USA, (1984) 81:7708] have reported the expression from CHO cells of a recombinant particle containing the pre-S(2) and S regions of HBsAg linked together in the order in which those sequences are found in nature. See also, U.S. Patent No. 4,722,840 to Valenzuela et al.

Rather than merely linking the T cell epitope to the B cell epitope, a composite polypeptide can be prepared (expressed) that contains a pre-S(2) T cell epitope polypeptide sequence of one or both subtypes in addition to the B cell epitope polypeptide sequences. Additionally, a composite polypeptide can be made with multiple T and B cell epitopes of each subtype by including two genomes for the same sequences that code for subtype variants such as the ad and av subtypes. In this way a plurality of pre-S(2) T cell epitope polypeptides of each subtype can
be operatively linked to a number of group- and subtype-specific B cell epitope polypeptides and expressed. However, use of a plurality of short polypeptide comprising not more than about 30 amino acid residues may find advantage over the use of the same polypeptides linked together to form a single larger polypeptide, as described hereinafter.

A recombinant polypeptide can be prepared using well known techniques of genetic engineering and commercially available reagents since the amino acid residue sequences and the genomic sequences for the pre-S and S regions of HBsAg are known. Vectors specifically designed for cloning a genome to provide quantities useful for subsequent expression of the desired amino acid residue sequence are also commercially available as are vectors specifically designed for expression. Bacteria, yeast and mammalian cells suitable for transfection by the expression vector and subsequent expression of the polypeptides are also commercially available.


Numerous methods of operatively linking polypeptide (a) to polypeptide (b) are known in the art and can be used when the composite polypeptide is not recombinantly expressed as a unit. For example, a
dialdehyde such as glutaraldehyde and a reductant such as sodium borohydride can be used to link amino-terminal residues of the two polypeptides. A cysteine residue can be added to a terminal residue of polypeptide (a). Using N-maleimidobenzoyl-N-hydroxy succinimide ester (MBS) following the procedure of Liu, et al, (1979) *Biochemistry*, 18:690, the cysteine residue can be covalently linked to the MBS-reacted amino-terminal residue of polypeptide (b). A water-soluble carbodiimide such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide can also be utilized to operatively link the two polypeptides.

C. Utility

The composite polypeptides of this invention find use as immunogens of an HBV vaccine when present in an effective amount in a physiologically acceptable diluent. The composite polypeptides prime T cell help relevant to native HBV. That is, the polypeptides prime T cells that recognize native HBsAg. Additionally, the composite polypeptides find use in inducing antibody production. Formulation of compositions for use as vaccines is well known and is described hereinafter.

As described in the examples, an immunogen comprising a pre-S(2) T cell epitope joined to the amino terminus of a native pre-S(2) B cell epitope was compared to a polypeptide corresponding to 120-145 [a pre-S(2) sequence said to be useful as a synthetic "vaccine"] and to a native pre-S(2) region sequence (p133-174). The p120-145 sequence induced antibody in approximately 50% of the murine strains examined and stimulated T cells. However, the T cells were only relevant to the peptide, not to native. That is, T cell proliferation was induced in response to the
peptide but not in response to HBsAg. In contrast, a composite polypeptide of this invention induced antibody in 100% of the strains examined and stimulated T cells in most strains that responded to HBsAg.

The native pre-S(2) region (i.e., p133-174) primed T cells relevant to native HBsAg and induced a small amount of antibody that reacted with the native B cell epitopes, 133-139 (the group-specific epitope) and 137-143 (the subtype-specific epitope). The majority of induced antibody reacted with residues 156-171, which sequence is not a native B cell epitope. In contrast, when using a composite polypeptide of this invention, (p151-174)-(p133-143), the majority of the antibody was directed to the native B cell epitopes, particularly the group-specific epitope, 133-139.

IV. Composite Subtype Immunogens

In another aspect, the present invention provides a composite subtype immunogen that contains an amino acid sequence that corresponds to at least one native HBsAg/α T cell epitope and an amino acid sequence that corresponds to at least one native HBsAg/γ T cell epitope. Those sequences are designated polypeptides (α1) and (α2), respectively. Preferably, the T cell epitope polypeptides from the pre-S region will be included.

The commercial HBsAg S region vaccines developed to date (plasma-derived and recombinant) have consisted of subviral particles of a single subtype (i.e., αd or αy). The rationale has been that antibodies to the group-specific (α) determinant(s) are protective, and subtype-specific antibodies are not required for protection. This practice has not
taken into account the desirability of priming subtype-specific T cell memory. Thus, when an individual was immunized with one subtype, subtype-specific memory T cells would be incapable of evoking an anamnestic response in the event of a subsequent viral infection with the heterologous subtype.

It has been previously demonstrated that a number of murine strains immunized with HBsAg/P25(ad) recognized subtype-specific T cell sites exclusively, and no group-specific sites within the S region, but nonetheless produced anti-\(\alpha\) antibodies as well as anti-\(\delta\) antibodies. Therefore, subtype-specific T cells are able to help group-specific B cell antibody production. Milich, et al, J. Immunol., 134:4194 (1985).

It has now been found that when murine strains B10.S and B10.M were examined for the influence of subtype on the pre-S(2) immune response, T cell recognition of the pre-S(1) and pre-S(2) regions was also found to be subtype-specific. That is, p39/ad-primed T cells recognized HBsAg/ad particles, but not HBsAg/av particles. Those studies are described in detail in the examples. This subtype-specificity was confirmed in the studies using synthetic peptides. Peptides of the \(\delta\) subtype invariably did not cross-react with \(\gamma\) subtype peptides at the T cell level.

Thus, the present invention contemplates a composite subtype immunogen comprising a first immunogenic polypeptide having a T cell epitope of the \(\delta\) subtype and a second immunogenic polypeptide having a \(\gamma\) subtype T cell epitope polypeptide. For use in the methods of this invention, the first and second polypeptides will each be present in an amount sufficient to induce a subtype-specific T cell
response. The polypeptides can be in the form of polypeptide particles such as the HBsAg/p25, /p33 or /p39 particles as discussed herein or as the pre-S(2) T cell epitope polypeptides or as composite polypeptides described herein. Both subtype T cell epitope polypeptides can also be present in a single polypeptide sequence as in the composite polypeptides discussed herein, except that such a polypeptide contains a T cell epitope polypeptide for each subtype that need not be a pre-S(2) T cell epitope.

Thus, the present invention contemplates a multiple HBV subtype particle comprising assembled polypeptides. The polypeptides that form the particle can be one or more of the composite polypeptides described herein, alone or in combination with one or more of the HBsAg/p25, /p33 and /p39 polypeptides. If the particle is comprised of one or more of the HBsAg/p25, /p33 and /p39 polypeptides, at least one of those polypeptides is of the d subtype and at least one is of the y subtype.

The multiple HBV subtype particles of the present invention can be prepared using well known recombinant DNA techniques, such as those described in U.S. Patent No. 4,722,840 to Valenzuela et al. For instance, when the particle contains a plurality of different polypeptides each of the polypeptides can be prepared separately and then admixed and maintained under conditions for particle assembly. Alternately, the different polypeptides can be expressed in the same cell, preferably under conditions for assembly therein. It is preferred that only one polypeptide for each subtype be expressed in a single cell, preferably in a polycistronic manner. For instance, HBsAg/p25 subtypes d and y can be dicistronically expressed in a yeast or bacterium to ensure that
approximately equivalent amounts of polypeptide of each subtype are assembled into the particle.

In a preferred embodiment, at least one of the T cell epitope polypeptides, usually both of the T cell epitope polypeptides, is joined to a native HBsAg B cell epitope polypeptide. T cell epitopes of each subtype may be attached to the same B cell epitope polypeptide. Usually however, the composite subtype immunogen will comprise polypeptides having T and B cell epitopes of a single subtype. The composite subtype immunogens include mixtures of HBsAg/p39 of each subtype. Alternatively contemplated are mixtures of short, chemically synthesized polypeptides, which include T and B cell epitopes of each subtype. Also contemplated are particulate structures, for example liposomes and the like, containing a B cell epitope(s) of interest exposed on the surface. These epitopes need not be present on the same molecule, that is, they can be intrastructural, not necessarily intramolecular.

The composite subtype immunogens of this invention find use as T cell immunogens to prime T cells that recognize native HBsAg. When the composite subtype immunogens include B cell epitopes, the immunogens find use as the immunogen of an HBV vaccine. Use of the immunogens is described in detail below.

V. Vaccines

A. Introduction

The word "vaccine" in its various grammatical forms is used herein to describe a type of inoculum containing one or more polypeptide of this invention as an active ingredient that is used to induce active immunity in a host mammal against HBV. Since active
immunity involves the production of antibodies, a vaccine or inoculum may thus contain identical ingredients, but their uses are different. In most cases, the ingredients of a vaccine and of an inoculum are different because many adjuvants useful in animals may not be used in humans.

B. Preparation

The preparation of a vaccine that contains polypeptide molecules as active ingredients is well understood in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation can also be emulsified.

The active immunogenic ingredient is dissolved, dispersed or admixed in an excipient that is pharmaceutically acceptable and compatible with the active ingredient as is well known. The phrases "suitable for human use" and "pharmaceutically acceptable" (physiologically tolerable) refer to molecular entities and compositions that typically do not produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Suitable excipients are, for example, water, saline, phosphate buffered saline (PBS), dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, carriers or adjuvants which enhance the effectiveness of the vaccine. A preferred embodiment contains about 1 mg to about 5 mg HBsAg homolog peptide, exclusive of carrier, in about 1 ml PBS.
C. Carriers

One or more additional amino acid residues may be added to the amino- or carboxy-termini of the synthetic polypeptide to assist in binding the polypeptide to a carrier. Cysteine residues added at the amino- or carboxy-termini of the synthetic polypeptide have been found to be particularly useful for forming polymers via disulfide bonds. However, other methods well known in the art for preparing conjugates can also be used. Exemplary additional linking procedures include the use of Michael addition reaction products, dialdehydes such as glutaraldehyde, Kliipstein et al., J. Infect. Dis., 147:318-326 (1983) and the like, or the use of carbodiimide technology as in the use of a water-soluble carbodiimide to form amide links to the carrier, as discussed before for linking a plurality of polypeptides together to form a synthetic multimer.

Useful carriers are well known in the art, and are generally proteins themselves. Exemplary of such carriers are keyhole hemocyanin (KLH), edestin, thyroglobulin, albumins such as bovine serum albumin (BSA) or human serum albumin (HSA), red blood cells such as sheep erythrocytes (SRBC), tetanus toxoid, cholera toxoid as well as poly amino acids such as poly (D-lysine: D-glutamic acid), and the like.

As is also well known in the art, it is often beneficial to bind a synthetic polypeptide to its carrier by means of an intermediate, linking group. As noted above, glutaraldehyde is one such linking group. However, when cysteine is used, the intermediate linking group is preferably an m-maleimidobenxoyl N-hydroxy succinimide (MBS), as was used herein.
Additionally, MBS may be first added to the carrier by an ester-amide interchange reaction as disclosed by Liu et al., supra. Thereafter, the addition can be followed by addition of a blocked mercapto group such as thiolacetic acid (CH$_2$COSH) across the maleimido-double bond. After cleavage of the acyl blocking group, a disulfide bond is formed between the deblocked linking group mercaptan and the mercaptan of the added cysteine residue of the synthetic polypeptide.

Other means of immunopotentiation include the use of liposomes and immuno-stimulating complex (ISCOM) particles. The unique versatility of liposomes lies in their size adjustability, surface characteristics, lipid composition and ways in which they can accommodate antigens. In ISCOM particles, the cage-like matrix is composed of Quil A, extracted from the bark of a South American tree. A strong immune response is evoked by antigenic proteins or peptides attached by hydrophobic interaction with the matrix surface.

This invention adds another to the repertoire of useful carriers, i.e., a synthetic carrier moiety comprising the HBV pre-S(2) sequence 148-174 operatively linked to one or more polypeptide immunogen.

The choice of carrier is more dependent upon the ultimate use of the immunogen than upon the determinant portion of the immunogen, and is based upon criteria not particularly involved in the present invention. For example, if an inoculum is to be used in animals, a carrier that does not generate an untoward reaction in the particular animal should be selected.
D. Administration

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycos or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the rage of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25-70%.

A viral antigen homolog can be formulated into a vaccine as a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the antibody) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such
amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of about one hundred micrograms to about one hundred milligrams, preferably about one to about 10 milligrams and more preferably about 5 milligrams active ingredient per kilogram bodyweight individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in one or two week intervals by a subsequent injection or other administration.

A vaccine can also include an adjuvant as part of the excipient. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) for use in laboratory mammals are well known in the art. Pharmaceutically acceptable adjuvants such as alum can also be used. An exemplary vaccine thus comprises one ml of phosphate buffered saline (PBS) containing about 1 mg to about 5 mg viral antigen homolog (polypeptide immunogen) adsorbed onto about 0.5 mg to about 2.5 mg of alum. A preferred vaccine comprises 1 ml of PBS containing 1 mg viral antigen homolog adsorbed onto 2.5 mg of alum.

VI. Methods of Use

The present invention contemplates methods of using the composite subtype immunogens of this invention to modulate responsiveness to HBV. The method comprises administering a composition
comprising at least one HBsAg/d T cell epitope polypeptide and at least one HBsAg/y T cell epitope polypeptide in a physiologically acceptable diluent. Each T cell epitope polypeptide is present in an amount sufficient to induce a T cell response. Preferably, a plurality of T cell epitopes of each subtype are administered.

In one embodiment, the composition is free from B cell epitopes and is used to stimulate T cells in a chronically infected patient. In chronic HBV carriers, the patient's T cells do not respond properly to HBV. Use of composite subtype T cell immunogens may stimulate the patient's T cells to modulate the patient's response to HBV.

In a preferred embodiment, a composite subtype immunogen of this invention is used to induce responsiveness to a HBV vaccine. The composite subtype immunogen is present as a composition in a physiologically acceptable diluent which can be administered prior to or together with the vaccine. Usually, the T cell epitope(s) will be joined to an HBsAg B cell epitope(s), which B cell epitope(s) can comprise the immunogen of the vaccine. However, the T cell epitopes need not be joined to a B cell epitope to prime T cell help for antibody production, as described in detail in the examples.

Usually the composition will contain a plurality of HBsAg B cell epitopes operatively linked to a plurality of HBsAg T cell epitopes of each subtype. In a preferred embodiment the composite subtype immunogen serves as the immunogen of an HBV vaccine and comprises a plurality of T cell epitope polypeptides operatively linked to a plurality of B cell epitope polypeptides. The B cell epitope polypeptides present in the composite subtype
immunogen preferably include a plurality of cross-reactive B cell epitopes from the various regions of HBsAg and one or more subtype-specific B cell epitopes for at least one, preferably each of the most common, HBV subtype.

In this way the composition will provide for priming of both B and T cells in a single administration. Conveniently, the composition will contain HBsAg/p39 particles of each subtype. However, compositions containing a plurality of short synthetic polypeptides comprising the T and B cell epitopes also are contemplated.

The following examples are intended to illustrate, but not limit, the present invention.

VII. EXPERIMENTAL
  A. Materials And Methods
     1. Materials
        The C57BL/10 (B10), B10.D2, B10.BR, B10.S, B10.M, SJL/J, C3H.Q, C3H and Balb/c inbred murine strains were obtained from the breeding colony at the Research Institute of Scripps Clinic, La Jolla, CA. Female mice between six and eight weeks of age at the initiation of the studies were used in all studies.
        Recombinant HBsAg/p33/av particles were provided by Dr. P. Tiollais (Pasteur Institute, Paris). Those particles were prepared from chinese hamster ovary (CHO) cells transfected with a plasmid containing the S gene and the pre-S region gene of the av subtype of HBV. The CHO-derived particles are composed of the S-encoded p25 plus the pre-S(2) and S-encoded p33 in a ratio of approximately 3 to 1, as described in Michel et al., Proc. Natl. Acad. Sci. USA., (1984) 81: 7708, which is incorporated herein by reference. HBsAg/p33/ad recombinant particles were
supplied by Angen.

Purified, serum-derived HBsAg/p33/ad particles were supplied by Angen. Purified, serum-derived HBsAg/p39/ad particles were provided by Dr. D. Peterson (Medical College of Virginia, Richmond, VA). Those particles were derived from the adv² subtype and contain p39 represented as approximately 8.2 percent of the constituent polypeptides. A plasma-derived HBsAg/p39/av preparation was also provided by Dr. Peterson.

Purified preparations of HBsAg/p25/ad were provided by Dr. R. Louis (Cutter Laboratories, Berkeley, CA). That preparation was purified by Cutter Laboratories from human plasma by a combination of standard procedures including ultracentrifugation, ammonium sulfate precipitation, pepsin digestion and gel chromatography. The resulting preparation was composed exclusively of the 25 kilodalton (kd) polypeptide (p25) and its glycosylated form (gp28).

Plasma-derived, purified HBsAg preparations representing both the ad and av subtypes and consisting of various proportions of p25, p33 and p39 were provided by Dr. J. Gerin (Georgetown University, Rockville, MD). The preparation containing the highest proportion of pre-S(1) (p39) was used predominantly throughout his study, and was designated HBsAg/p39.

There was variation in the amount of S region-specific antigenicity per unit weight of particles in the HBsAg preparations utilized herein. Therefore, all HBsAg preparations were first equilibrated with respect to S region-specific antigenicity in terms of antibody binding using a solid phase, sandwich enzyme-linked immunosorbent assay (ELISA) described hereinafter. Then the
proportions of p25, p33 and p39 present in the various HBsAg particle preparations were similarly determined. In a sandwich ELISA assay, which is well known in the art, the antigen (HBsAg) to be measured was sandwiched between a solid phase-bound monoclonal antibody specific for either S, pre-S(1) or pre-S(2) and a peroxidase-labelled second anti-S monoclonal (non-cross-reactive with the solid phase anti-S monoclonal) used herein as a probe.

Two monoclonal antibodies (Mabs) specific for the S region of HBsAg were provided by Dr. P. Kaplan (Ortho Diagnostics, Inc., Raritan, NJ). Mab specific for the pre-S(1) region of HBsAg (A18/7) were provided by Dr. W. Gerlich (University of Gottingen, Gottingen, Germany). Those Mabs are described in Heerman et al., (1984) J. Virol., 52: 396. Mab 5520, 5521 and 5535 specific for pre-S2 were produced following immunization of Balb/c mice with the synthetic peptide p133-151 (subtype ayw) coupled to ovalbumin and were provided by Dr. T. Nakamura (Institute of Immunology, Tokyo, Japan), as described in Okamoto, et al, J. Immunol., (1985) 134: 1212. Mab 4408 and Mab 5161 specific for pre-S2, produced following immunization of Balb/c mice with intact p33 polypeptide derived from HBsAg/ayw particles as described by Machida, et al, (1984) Gastroenterol., 86: 910, were also provided by Dr. T. Nakamura.

2. Methods
   a. Immunization

To study in vivo antibody production, groups of mice were immunized using inocula containing either 4.0 micrograms of native HBsAg in its various forms, p25, p33 or p39, or 100 micrograms of the various synthetic polypeptides as immunogen dispersed in
complete Freund's adjuvant (CFA) by intraperitoneal (IP) injection as the primary immunization (1'). A secondary immunization (2') using an inoculum containing 2 micrograms (µg) of HBsAg in its various forms or 50 µg of a synthetic polypeptide as immunogen in incomplete Freund's adjuvant (IFA) was administered IP about four weeks after primary immunization (1'). Mouse sera were obtained from the retroorbital plexus 10 and 24 days following primary immunization and again 2 weeks following secondary immunization, unless otherwise stated.

In vivo priming for the T-cell proliferative assay was accomplished by hind footpad injections of either 4 µg of HBsAg or 100 µg of synthetic polypeptide in CFA.

b. Measurement of Anti-HBs And Anti-pre-S Antibodies

Anti-S and anti-pre-S antibodies induced by immunization with a synthetic polypeptide or native HBsAg, and anti-polypeptide antibodies induced by polypeptide immunizations were measured by the following methods.

(1) Radioimmunoassay

Murine sera were evaluated for anti-S, anti-pre-S(1) or anti-pre-S(2) and anti-synthetic polypeptide reactivity in an indirect, immunoglobulin class-specific, radioimmunoassay (RIA) utilizing as the solid phase-bound ligand either a synthetic polypeptide (1-2 µg per well), or purified HBsAg particles (0.1 micrograms per well). Goat anti-mouse IgG, were utilized to bind the murine antibodies bound to the solid phase ligand. After rinsing away any unbound goat anti-mouse antibodies, 125I-labelled swine
anti-goat IgG were admixed and maintained with the goat anti-mouse antibodies bound to the solid phase as described in Milich, et al, (1982) J. Immunol., 129: 320. HBsAg/p25/ad was used as a solid phase-bound ligand to assay anti-S-region specific antibody. Synthetic polypeptide p120-145, p133-140 or p135-145 was used as a solid phase-bound ligand to assay pre-S(2)-specific antibody. Similarly, synthetic polypeptide p94-117 was used to assay pre-S(1)-specific antibody. Other HBsAg preparations and synthetic peptides were used as solid phase-bound ligands in these studies as indicated. Data are expressed as an antibody titer representing the highest dilution to yield three times the counts of pre-immunization sera values. Monoclonal antibodies specific for these same antigens were assayed by the same indirect solid phase RIA except that the data are expressed as antibody titer representing the minimum concentration required to detect binding greater than five times the counts of background value.

(2) ELISA

Human sera were assayed for antibody reactivity using specific pre-S(1) or pre-S(2) synthetic peptides as solid phase-bound ligands (1.0 microgram per well) in an enzyme-linked immunosorbent assay (ELISA). Assayed sera were first admixed and maintained (incubated) with the solid phase-bound ligands. After removing any unbound serum constituents, the resulting solid phases were probed with a peroxidase-labelled anti-human IgG monoclonal antibody. Sample optical density values at 490 nanometers (O.D. 490) greater than four times that of normal sera were considered positive using 1:50 dilutions of human sera that were admixed and
maintained (incubated) with ligand. Pre-S(2) peptides used as ligands are listed below.

<table>
<thead>
<tr>
<th>Pre-S(2) T Cell Peptides Available and Used for Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>120-145/d &amp; y 143-174/d 156-169/d</td>
</tr>
<tr>
<td>133-174/d &amp; y 1440160/d 156-170/d</td>
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<tr>
<td>136-155/d &amp; y 146-157/d 156-172/d</td>
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<tr>
<td>146-165/d &amp; y 146-160/d 120-131/adw</td>
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<tr>
<td>156-174/d &amp; y 148-174/d 120-132/adw2</td>
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<tr>
<td>159-174/d &amp; y 149-170/d 120-145/adw2</td>
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<tr>
<td>161-174/d &amp; y 152-172/d 120-145/ayw</td>
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<tr>
<td>162-174/d &amp; y 153-174/d 128-138/adw2</td>
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<td>164-174/d &amp; y 154-170/d 141-174/d-(133-140)</td>
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<tr>
<td>166-174/d &amp; y 154-172/d 151-174/d-(133-143)</td>
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<tr>
<td>131-174/d 156-165/d</td>
</tr>
<tr>
<td>138-174/d 156-167/d</td>
</tr>
</tbody>
</table>

Antibody-antibody competition assays were performed by pre-admixing and maintaining (pre-incubating) varying concentrations [0.6 - 625 nanograms per milliliter (ng/ml)] of the unlabelled competing antibody with a predetermined limiting concentration of solid phase-bound ligand (HBsAg/p33, 0.1 ug well) for a time period of about 18 hours (overnight) at 4°C, followed by admixing and maintaining (incubating) of a predetermined amount of enzyme-labelled (horseradish peroxidase) second antibody whose inhibition was being measured. The results are expressed as percent inhibition of the labelled antibody solid phase-bound ligand interaction. Monoclonal antibodies 4408, 5521 and 5161 were labelled with enzyme and used as described. The competing antibodies used in these studies were
either monoclonal or polyclonal antibodies raised to either synthetic polypeptides or HBSAg particle preparations.

5  c.  HBSAg-Specific T Cell Proliferation Assay

Groups of four mice were immunized in the hind footpads with an emulsion of CFA and either 4 ug of HBSAg or 100 ug of synthetic polypeptide. Eight to ten days later, popliteal lymph node (PLN) cells were harvested and cultured in vitro to a concentration of 5x10^5 cells/ml with various challenge antigens (ligands). The in vitro antigens included native HBSAg [p25, p33 and p39 which contained various, known, ratios of S, pre-S(1) and pre-S(2)], and the pre-S region synthetic polypeptides p120-145/ad, p120-145/av.

Draining popliteal lymph node cells were aseptically removed from each mouse and teased to yield a single cell suspension. The cells were washed twice with a balanced salt solution (BSS) containing phosphate-buffered saline (pH 7.2). The cells were resuspended in Click's medium containing BSS, L-glutamine, sodium pyruvate, antibiotics, 2-mercaptoethanol, essential and non-essential amino acids and vitamins. [See Click, et al, (1972) Cell. Immunol., 3:264.] Click's medium was modified by the addition of 10 millimolar (mM) HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) and gentamycin (10 ug/ml), and by the substitution of 0.5 percent syngeneic normal mouse serum for fetal calf serum.

The antigens were assayed in culture over a dose range of 0.003-1 ug/ml for HBSAg preparations, and 0.07-200 ug/ml for synthetic polypeptides.
Viable lymph node cells (5x10^5) in 0.1 ml of medium were placed in flat-bottom microtiter wells (Falcon 3072, Falcon Plastics, Inc.) with: (a) 0.1 milliliter of medium containing various concentrations of a HBsAg preparation or a synthetic peptide, (b) culture medium and ovalbumin [200 micrograms per milliliter (µg/ml)] as a negative control, or (c) purified protein derivative (PPD) 100 µg/ml; (Parke-Davis, Detroit, MI) as a positive control.

Cultures were maintained for five days at 37°C in a humidified atmosphere containing 5 percent carbon dioxide in air.

On the fourth day, each culture was admixed and maintained (incubated) with one microcurie (µCi) ³H-thymidine (³HtdR) (6.7 Ci/millimole, New England Nuclear, Boston, MA) for 16 hours before harvesting. Cells were harvested onto filter strips and proliferation was determined by the incorporation of ³HtdR into DNA. The data are expressed as counts per minute (cpm) corrected for background proliferation in the absence of antigen. It was demonstrated previously that the HBsAg-specific proliferation response of draining PLN cells harvested up to 13 days post-immunization was due to proliferating T cells; Milich, et al, (1983) J. Immunol., 130:1401. Therefore, unfractionated PLN cells were used in analyses reported herein.

d. Synthesis Of Polypeptides

The polypeptides corresponding to the pre-S(2) region utilized herein were chemically synthesized by solid phase methods as described in Merrifield, et al, (1963) J. Am. Chem. Soc., 85:2149. A polypeptide for use in the present invention is designated below both by a residue number
abbreviation and by the polypeptide's amino acid residue sequence:

<table>
<thead>
<tr>
<th>Residue Numbers</th>
<th>Amino Acid Residue Sequence</th>
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</thead>
<tbody>
<tr>
<td><strong>POLYPEPTIDES</strong></td>
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<tr>
<td><strong>Subtype adw</strong></td>
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</tr>
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<td>136-174</td>
<td>VRGLYPAGGSGSGTVNPAPNIAHISISSISARTGDPVTI</td>
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<tr>
<td>136-155</td>
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<tr>
<td>148-159</td>
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</tr>
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<td>148-165</td>
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<tr>
<td>156-165</td>
<td>TTASPLSSIF</td>
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<tr>
<td>159-169</td>
<td>SPLSSIFSRIG</td>
</tr>
<tr>
<td>161-174</td>
<td>LSSIFSRIGDPALN</td>
</tr>
</tbody>
</table>

The solid phase method of polypeptide synthesis was practiced utilizing a Vega 250 Peptide
Synthesizer and an Applied Biosciences 430A Peptide Synthesizer, available commercially from Vega Biotechnologies, Inc., Tucson, AZ, and Applied Biosystems, Foster City, CA, respectively. The composition of each polypeptide was confirmed by amino acid analysis.

Briefly, in preparing a synthetic polypeptide by the above solid phase method, the amino acid residues are linked to a resin (solid support) through an ester linkage from the carboxy-terminal residue. When the polypeptide is to be linked to a carrier or another polypeptide via a Cys residue or reacted via terminal Cys residues, it is convenient to utilize that Cys residue as the carboxy-terminal residue that is ester-bonded to the resin.

The alpha- amino group of each added amino acid is typically protected by a tertiary-butoxycarbonyl (t-BOC) group prior to the amino acid being added into the growing polypeptide chain. The t-BOC group is then removed prior to addition of the next amino acid to the growing polypeptide chain.

Reactive amino acid side chains were also protected during synthesis of the polypeptides. Usual side-chain protecting groups were used for the remaining amino acid residues as follows: O-(p-bromobenzyloxy carbonyl) for tyrosine; O-benzyl for threonine, serine, aspartic acid and glutamic acid; 4-methylbenzl and S-methoxybenzyl for cysteine, dinitrophenyl for histidine; 2-chlorobenzoxy carbonyl for lysine and tosyl for arginine.

by Merrifield, et al., (1963) *J. Amer. Chem. Soc.* 85:2149 was used to synthesize the peptides from the Vega 250 Peptide Synthesizer. Repeat coupling of the incoming protected amino acid was sometimes necessary to effect complete coupling efficiency. All coupling reactions were more than 99% complete by the quantitative ninhydrin test of Sarin (1981), *Analytical Chemistry* 117:147.

After preparation of a desired polypeptide, a portion of the resulting, protected polypeptide (about 1 gram) was treated with two milliliters of anisole, and anhydrous hydrogen fluoride, about 20 milliliters, was condensed into the reaction vessel at dry ice temperature to form an admixture. The resulting admixture was stirred at about 4°C for about one hour to cleave the protecting groups and to remove the polypeptide from the resin. After evaporating the hydrogen fluoride at a temperature of 4°C with a stream of N₂ the residue was extracted with anhydrous diethyl ether three times to remove the anisole, and the residue was dried.

The dried material was extracted with 5 percent aqueous acetic acid (3 times 50 milliliters) to separate the free polypeptide from the resin. The extract-containing solution was lyophilized to provide the polypeptide.

The polypeptides derived from the pre-S(1) region were chemically synthesized. The resultant synthetic polypeptides were used as reagents in an enzyme-linked immunosorbent assay (ELISA) to detect anti-HBsAg antibodies. The synthetic polypeptides were also used in inocula, usually in CFA orIFA, as discussed hereinbefore.

**EXAMPLE 1**

T Cell Response To HBsAg Is Subtype Specific
The influence of subtype on the pre-S(2) immune response was studied. Two murine strains, B10.S (an S-region nonresponder) and B10.M (a pre-S(2)- and S-region nonresponder), were examined. The mice were primed with 6.0 ug of HBsAg/p39ad and HBsAg/p39ay. The T cell proliferative response to varying amounts of HBsAg/p39ad was studied.

The results are illustrated in Figure 2. As seen in the figure, p39/ad-primed T cells recognized pre-S(2)/ad-containing HBsAg particles, but not pre-S(2)/ay-containing particles. Therefore, T cell recognition of the pre-S(2) region is subtype-specific. Studies with pre-S(1) region T cell sites (i.e., p12-21 and p53-73) also indicate a subtype-specific T cell response within the pre-S(1) region.

Furthermore, B10.M mice which are nonresponsive to the pre-S(2) and S regions when immunized with pre-S(2) or S-HBsAg/ay particles, are responsive to pre-S(2)-HBsAg/ad particles and produce both anti-S and anti-pre-S(2) antibodies, as illustrated in Table 3. As can be seen from Table 3, below, B10.M mice, which are nonresponsive to the pre-S(2) and S regions when immunized with pre-S(2) or S-HBsAg/ay particles, are responsive to pre-S(2)-HBsAg/ad particles, and produce both anti-S and anti-pre-S(2) antibodies.

<table>
<thead>
<tr>
<th>IMMUNOGEN</th>
<th>STRAIN</th>
<th>TIME</th>
<th>ANTI-S</th>
<th>ANTI-PRE-S(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg/P33(ay)</td>
<td>B10.S</td>
<td>10d.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24d.</td>
<td>0</td>
<td>1:640</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>1:640</td>
<td>1:10,240</td>
</tr>
<tr>
<td></td>
<td>B10.M</td>
<td>10d.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24d.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE 3
SUBTYPE SPECIFICITY OF ANTIBODY RESPONSES

ANTIBODY TITER
HBsAg/P33(ad) B10.S 10d. 0 1:640
   24d. 1:640 1:640
B10.M 10d. 0 1:160
   24d. 1:2560 1:5120

1  B10.S mice are nonresponsive to the S region of HBsAg of both subtypes when immunized with HBsAg/P25. B10.M mice are nonresponsive to the S region of HBsAg of both subtypes (i.e., HBsAg/P25), and to the pre-S(2) region of the ay subtype [i.e., HBsAg/gP33(ay)], but are responsive to the ad subtype [i.e., HBsAg/gP33(ad)].

2  Time in days (d.) after primary immunizations. 2= response after secondary immunization.

These results indicate that S- and pre-S(2)-containing HBsAg vaccines benefit from inclusion of both pre-S(2) and S ad and ay subtypes for at least two reasons: (1) the priming of T cell memory relevant to subtype infection with either subtype of the virus; and (2) nonresponders to one subtype can be responsive to the other subtype in terms of antibody production and therefore, the number of genetic nonresponders can be reduced.

This study demonstrates that the priming of T cell memory is subtype specific. The study also demonstrates that an animal which is a nonresponder to one HBsAg subtype may be responsive to the other subtype in terms of antibody production. Taken together, the results demonstrate that including T cell epitopes of both subtypes reduces the number of genetic nonresponders.
EXAMPLE 2
Determination of Pre-S(2) T Cell Epitopes

The fine specificity of T cells specific for the pre-S(2) region of HBSAg was mapped as described for the pre-S(1) region. (See Milich, et al, J. Immunol., 138:4457-4465, 1987) Briefly, various inbred, H-2 disparate murine strains were immunized with 6ug of HBSAg/p33 particles of the ad subtype. Ten days later, lymph node T cells were harvested and cultured with varying concentrations of HBSAg/p33 particles of both ad and ay subtypes; and a panel of synthetic peptides (see list of pre-S(2) T cell peptides).

The reciprocal experiment was performed when active T cell peptides were localized. Mice were immunized with synthetic peptides and lymph node T cells were harvested and cultured with native HBSAg (p33) particles to confirm the relevance of the peptide T cell response to native protein.

Table 2 illustrates the pre-S(2) T cell epitopes for the various mouse strains tested. As shown in the table, the T cell epitopes for the adv subtype are found from residues 148 through 174, while the ayw T cell epitopes are from residues 136 through 174. The table also illustrates that the B10.M strain did not respond to any portion of the ay pre-S(2) sequence as a T cell epitope, but did recognize a pre-S(2)/ad T cell epitope.

This study further illustrates the need for including T cell epitopes of each subtype to reduce the number of genetic nonresponders to an HBV vaccine.

EXAMPLE 3
Response to Synthetic Pre-S(2) Vaccine

Synthetic polypeptides having a sequence corresponding to p133-174/ad or p131-174/ad were prepared and injected into mice as described above. The induced antibody was analyzed by ELISA as described above. Although a small amount of the induced antibody was directed to p133-139 and p137-143 (the native group and subtype-specific B cell epitopes, respectively), the majority of the antibody response was to p156-171, which is not a native B cell epitope.

To determine whether the orientation of the T and B cell epitopes influenced the response, mice were injected with (p141-174)-(p133-140)/ad or (p151-174)-(p133-143)/ad. The induced antibody was studied. Both polypeptides were found to be more immunogenic than p133-174. That is, more antibody was produced. Further, most of the antibody was directed to p133-139, the native, group-specific B cell site. Some antibody was also directed to p137-143, the native subtype-specific B cell epitope. Further, an increased amount of native antibody in comparison to the amount induced by p133-174 (a portion of the pre-S(2) region in its natural order) was produced.

The use of those peptides was compared to p120-145, a polypeptide which has been used as a synthetic pre-S(2) vaccine. In contrast to p120-145 where about 50% of mouse strains tested were nonresponders in terms of antibody production, no murine strains were nonresponders when immunized with either (p141-174)-(p133-140)/ad or (p151-174)-(p133-143)/ad.

Furthermore, (p151-174)-(p133-143) was more effective as a pre-S(2) vaccine than p120-145 in that it primed T cells relevant to native HBsAg. In
contrast, p120-145 primed T cells relevant to the polypeptide immunogen but not to HBsAg.

Therefore, an enhanced T cell region may be necessary to eliminate nonresponders. To enhance the T cell region, either a larger region which includes more of the T cell epitopes may be used, i.e. p141-174 rather than p151-174, or a plurality of smaller T cell epitope polypeptide regions may be used concurrently (e.g., (p146-160)-(p133-143), (p151-165)-(p133-143) and (p160-174)-(p133-143)).

As shown previously, antibody production to the three regions of HBsAg is independently H-2 restricted and yet exhibits overlapping regulatory mechanisms. The influence of region-specific T cell recognition of HBsAg, which could function to help B cell clones responsive to all regions was directly demonstrated by murine B10.M strain T cells which do not respond to the S or pre-S(2)/av regions. When the strain was immunologically primed with HBsAg/p39av, the T cells recognized only the pre-s(1) region. However, this strain produces anti-S-, anti-pre-S(2)- and anti-pre-S(1)-specific antibodies following HBsAg/p39 immunization. Therefore, pre-S(1)-specific T cells can provide functional help for B cell clones that recognize S, pre-S(2), and pre-S(1) region determinants, presumably on the p39 polypeptide of HBsAg.

The cumulative results of the current studies on the immune response to the pre-S regions of HBsAg have important implications in terms of future HBsAg vaccine development. Previous work suggested an unexpectedly low number of T cell recognition sites on HBsAg/p25 considering its particulate nature and the size of the subunit polypeptide (226 amino acid
residues); Milich, et al, (1985) J. Immunol., 134:4203. This was reflected in the ability to identify at least two murine H-2 haplotypes (H-2\textsuperscript{k},\textsuperscript{t}) that were total nonresponders, and one (H-2\textsuperscript{t}) that was only responsive to subtype determinants following HBsAg/p25 immunization; Milich, et al, (1984) J. Exp. Med., 159:41. In contrast, a nonresponding haplotype following HBsAg/p39 immunization has not yet been identified amongst the following H-2 haplotypes studied to date: q, d, s, k, b, p, f, t4.

As discussed above, the lack of genetic nonresponsiveness to HBsAg/p39 can be attributed to an increased complexity of T cell recognition. At the T cell level, the pre-S(2) and pre-S(1) regions provide additional subtype-specific antigenic heterogeneity to HBsAg/p25, which comprises the current plasma-derived, American vaccine (Heptavax-B, Merck Sharp and Dohme, Philadelphia, PA). Therefore, inclusion of T cell epitopes of both subtypes of HBsAg/p39 in HBsAg vaccines can decrease the incidence of genetic nonresponsiveness.

It is clear from the present work and previous studies (Neurath, et al, (1984) Science, 224:392, Neurath, et al, (1985) Nature (London), 315:154) that pre-S(1) and pre-S(2)-specific, as well as S-specific, antibodies are produced during HBV infection in man. However, production of specific antibodies to the pre-S(1), pre-S(2) and S regions of HBsAg are not predictive of specific T cell responses since T cell help may derive from a population of T cells limited to recognition of a single region of a particular subtype. This is important in the context of the hypothesis that HBV clearance is mediated at the T cell level. For example, a pre-S(2)-specific helper T cell or cytotoxic T cell response may be
required for viral clearance, but not required for
anti-pre-S(2) antibody production as was observed in
the B10.M strain.

It has been reported that a number of
HBsAg/p25 vaccine nonresponders that were subsequently
infected with HBV not only cleared the infection (i.e.
did not become chronic carriers), but also produced an
Transfusion, 21:601 (Abstract). One possible
explanation of this phenomenon is that upon infection
with HBV, pre-S-specific T cells provided help for S
region-specific B cells resulting in anti-S production
by vaccine nonresponders, analogous to the B10.S and
B10.M murine models. Therefore, the use of a
sufficient variety of T cell epitopes of each subtype
may eliminate human nonresponders as was demonstrated
in mice.

EXAMPLE 4

Studies using a truncated pre-S(2)
polypeptide and short synthetic peptides showed that
the terminal sequence (p148-174) is the dominant focus
of T cell recognition in all murine strains tested.
This truncated polypeptide sequence contains 17
subtype specific recognition sites, the fine
specificity of which is dependent on the haplotype of
the responding strain. The ability of synthetic
peptides to elicit T cells crossreactive with the
native pre-S(2) region is variable and dependent on
the nature of the immunizing peptide.

Two panels of peptides were synthesized. The
first panel containing the d and y subtypes of the
pre-S(2) region included:

p133-174, p148-174, p120-145, p136-155, p141-
160, p146-165, p151-170, p156-174, p161-174,
p166-174.
The second panel included:
T cell responses were assayed as described hereinbefore.

Results
T cell proliferative response to the pre-S(2) region of HBsAg is subtype-specific

Figure 3 shows that T cell proliferative response to the pre-S(2) region of HBsAg is subtype-specific. B10.s and B10.M mice were immunized with HBsAg/P33 of the d or y subtype and draining popliteal lymph node (PLN) T cells were examined for proliferative response to HBsAg/P33 and HBsAg/P25 of both subtypes. As shown in Figure 3A, T cells in B10.S mice primed with HBsAg/P33d responded to the d subtype only. The pre-S(2) specificity of the response was confirmed by the lack of response to HBsAg/P25 d or y particles, which lack the pre-S(2) region. The reciprocal response is shown in Figure 3B wherein the mice primed with the y subtype responded efficiently to HBsAg/P33y but only slightly to HBsAg/P33d.

In contrast, although subtype-specific, Figure 4 shows the response in B10.M mice to be only marginal to the y subtype of HBsAg/P33.

C-terminal half of pre-S(2) is the dominant T cell recognition site for all murine strains tested.
Figure 5 demonstrates that T cell recognition in a variety of strains is focused on the C-terminal half of the pre-S(2) region. HBsAg/P33d-primed T cells from a number of strains were cultured with full length (P33) or N-terminally truncated (P28) HBsAg particles. Because the truncated version elicited significant T cell proliferation in all strains immunized with HBsAg/P33, it appears that the N-terminal residues (120-139) do not play a significant role in T cell recognition.

Fine specificity of T cell recognition of the pre-S(2) region of HBsAg is MHC-dependent. Peptide 133-174, possessing T and B cell recognition sites relevent to the native protein and corresponding to the d and y subtypes was used to further define T cell recognition sites within the pre-S(2) region. Various H-2 congenic strains were immunized with the d and y subtypes of this peptide and draining PLN cells were examined for proliferative response elicited by a panel of antigens.

Although again showing subtype specificity, the fine specificity of B10 T cell recognition was distinct for the d and y subtypes. As seen in Figure 6A, the sequences predominantly recognized by primed B10 T cells were p141-160d, p146-165d and, to a lesser degree, p151-170d. In contrast, for the y subtype, p136-155y was the dominant recognition site and to a lesser degree, p151-165y (Figure 6B). Therefore, the responses are not crossreactive because distinct regions are recognized by T cells primed with each subtype. This suggests substitution of agreptic residues (residues which contact MHC molecules) relevant to the H-2b haplotype.

On the other hand, B10.M p133-174d primed T cells responded to p156-170d and p156-174d (Figure
7A), and completely failed to respond to p133-174y (Figure 7B), which is consistent with Figure 4. It appears that one or several of the 6 amino acid substitutions in this area (Figure 10) of the y subtype interferes with reaction of agrotopic residues with B10.M MHC class II molecules.

Similar subtype specificity is evident for B10.S as shown in Figure 8A and B. Immunization with p133-174d elicited T cell responses to p151-170d and 156-174d but not their y counterparts (6A). Conversely, immunization with p133-174y elicited a vigorous response sites 156-174y and 161-174y.

The d subtype of p133-174 is only moderately immunogenic in B10.D2, as seen in Figure 9A, in contrast with the y subtype (Figure 9B), where peptides are also antigenic. The p146-165y sequence appears to represent the focus of the response.

A summary of T cell recognition of the pre-S(2) region of the d and y subtypes in 8 murine strains representing 8 different H-2 haplotypes is presented in Figure 10. It is clear from the above analysis that the specific T cell site recognized is dependent on the H-2 haplotype of the responding strain. The sheer number of overlapping but distinct T cell recognition sites (17) and their concentration within such a limited region (27 residues) of pre-S(2) is striking. It is also noteworthy that every T cell recognition site defined in this study is subtype-specific. This is consistent with B10.S and B10.M T cell response specificity to HBsAg/P33 and indicates that the latter strains are not unique in this regard.

In Figure 11A, B10 HBsAg/P33-primed T cells react efficiently with HBsAg/P28 (the truncated sequence), p141-160 and p146-165, consistent with the dominant T cell site, p149-157, which was also defined
with synthetic peptides p133-174 and p148-174 as immunogens. Similarly, B10.M, HBsAg/P33-primed T cells react efficiently with HBsAg/P28, p151-170, and p156-174, consistent with the dominant site p156-167, which was also defined with synthetic peptides p133-174 and p148-174 as the immunogens (Figure 11B). There is no evidence that HBsAg/P33-primed T cells of any strain recognize the N-terminal half (p120-147) of the pre-S(2).

Discussion

These studies suggest the possibility that regions of a protein that possess physical/chemical characteristics ideal for interacting with MHC molecules and/or TCR will be excellent targets for cell recognition by multiple strains. Although a "universal" T cell recognition site probably does not exist, the p148-174 region of pre-S(2) represents a relatively short sequence (27 residues) with 17 specific sites recognized uniquely by 8 of 8 H-2 haplotypes tested.

As an additional benefit, the p148-174 sequence of the pre-S(2) region may serve as a synthetic, aspecific T cell carrier moiety for non-HBV B cell epitopes to provide Th cell function for induction of antibody production in a variety of MHC haplotypes.

If the human T cell response to pre-S(2) is focused on the subtype variable C-terminal region, which seems likely in view of the predominance of this region in murine T cell recognition, both major subtypes of HBsAg/P33 should be included in third generation pre-S(2)-containing vaccines. Also, it is imperative that a synthetic subunit vaccine be capable of eliciting T cells that can crossreact with the
native antigen. As was seen in this study, and below, this feature is dependent upon the nature of the synthetic immunogen.

EXAMPLE 5

Synthetic Pre-S(2) Immunogens

One purpose of this study was to examine the influence of viral subtype on \textit{in vivo} antibody production to the pre-S(2) region of HBsAg. A second purpose was to "engineer" a synthetic pre-S(2) immunogen based on combining the dominant B and T cell recognition sites into a single peptide. Methods used were as stated hereinabove.

Results

HBsAg/P33 Particles as Immunogen

Table 4 shows the primary antibody response elicited by immunization with HBsAg/P33 particles in six strains of mice varying in H-2 haplotype.
Table 4.
Primary antibody response to immunization with HBsAg/P33d.

<table>
<thead>
<tr>
<th>STRAIN*</th>
<th>TIME (DAYS)</th>
<th>H-2</th>
<th>ANTIBODY TITER (1/DILUTION)</th>
<th>Anti-pre-S(2)</th>
<th>Anti-S</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>B10</td>
<td>10</td>
<td>b</td>
<td>2,560 (+)</td>
<td>160 (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>40,960 (+)</td>
<td>2,560 (+)</td>
<td></td>
</tr>
<tr>
<td>B10.M</td>
<td>10</td>
<td>f</td>
<td>160 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>5,120 (0)</td>
<td>2,560 (0)</td>
<td></td>
</tr>
<tr>
<td>B10.S</td>
<td>10</td>
<td>s</td>
<td>640 (+)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>1,280 (+)</td>
<td>640 (±)</td>
<td></td>
</tr>
<tr>
<td>B10D2</td>
<td>10</td>
<td>d</td>
<td>1,280 (+)</td>
<td>40 (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>2,560 (+)</td>
<td>10,240 (+)</td>
<td></td>
</tr>
<tr>
<td>B10 BR</td>
<td>10</td>
<td>k</td>
<td>40 (0)</td>
<td>0 (0)</td>
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</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>2,560 (+)</td>
<td>320 (+)</td>
<td></td>
</tr>
<tr>
<td>C3H.Q</td>
<td>10</td>
<td>q</td>
<td>640 (+)</td>
<td>40 (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>10,240 (+)</td>
<td>640 (+)</td>
<td></td>
</tr>
</tbody>
</table>

* Groups of 5 mice each of the indicated strains were immunized i.p. with 2.0 μg of HBsAg/GP33d emulsified in CFA. Ten and 24 days post-immunization sera was collected, pooled and IgG anti-pre-S(2) and anti-HBS (S) antibody was determined by ELISA using the pre-S(2) peptide p120-145d and HBsAg/P25d as solid-phase antigens, respectively. Antibody titer is expressed as the reciprocal of the serum dilution which yielded 3x the O.D. reading of preimmunization sera.

b Responder status after immunization with the ayw subtype of HBsAg/P33.
Previous studies have shown the B10.M strain to be nonresponsive to both the S and pre-S(2) regions of HBsAg. However, these studies used HBsAg particles of the ayw subtype only. When challenged with HBsAg particles of the adw₂ subtype, anti-pre-S(2) and anti-S region antibodies were produced. These positive responses are consistent with the finding that B10.M T cells are capable of efficient recognition of the adw₂ subtype but not the ayw subtype of the pre-S(2) region. Since the T cell studies also indicated that the B10.M strain was T cell nonresponsive to the S region of both subtypes, the antibody production likely results from pre-S(2)/d-specific T help (Th) cell function. These studies indicate that the frequency of nonresponders to pre-S(2) can be decreased simply by immunizing with both subtypes.

Table 5 shows the fine specificity for anti-pre-S(2) antibody elicited by immunization with HBsAg/P33d. The conformation-independent nature of pre-S(2) region antigenic determinants allows the use of synthetic peptides as ligands for specificity analysis. All strains produced antibodies against the p120-145 sequence, regardless of subtype.
Table 5
Fine specificity of anti-pre-S(2) antibody elicited by immunization with HBsAg/P33d.

<table>
<thead>
<tr>
<th>STRAIN*</th>
<th>ANTIBODY TITER (1/DILUTION)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANTIGENS</td>
</tr>
<tr>
<td></td>
<td>p120-145d</td>
</tr>
<tr>
<td>B10</td>
<td>40,960</td>
</tr>
<tr>
<td>B10.M</td>
<td>5,120</td>
</tr>
<tr>
<td>B10.S</td>
<td>1,280</td>
</tr>
<tr>
<td>B10.D1</td>
<td>2,560</td>
</tr>
<tr>
<td>B10.BR</td>
<td>2,560</td>
</tr>
<tr>
<td>C,H,Q</td>
<td>10,240</td>
</tr>
</tbody>
</table>

Groups of 5 mice each of the indicated strains were immunized i.p. with 2.0 µg of HBsAg/P33d emulsified in CFA, and 24 days later IgG antibody specific for the indicated pre-S(2) region peptides was determined by ELISA. Antibody titer is expressed as the reciprocal of the serum dilution which yielded 3x the O.D. reading of preimmunization sera.
The lack of reactivity with p120-132d and positive reactivity with p133-140d/y defines the dominant specificity to be 133-140 (the higher p125-145 titres are due to greater adhesion of this peptide to a solid phase surface). The p133-140 sequence is conserved between the d and y subtypes, therefore, the lack of subtype-specific antibody reactivity on the p120-145 peptide (i.e., the binding to y and d subtypes is not significantly different). These, and similar findings after HBsAg/P33y immunization, indicate that the predominant antibody response to the pre-S(2) region is group-specific. Therefore, the influence of subtype is expressed at the level of antibody responder status, which is T cell mediated, and is not apparent in terms of antibody crossreactivity between the d and y subtypes. That is, viral subtype influences whether specific antibody is produced, but once produced, the antibody is fully crossreactive on both subtypes.

**Synthetic Immunogen p133-174/d**

The p133-174 peptide was very immunogenic to its own sequence, as seen in Table 6, however, antisera produced crossreacted rather poorly with native HBsAg/P33d, with the exception of strain B10. Although the p133-174d sequence is that of the native HBsAg/P33d, the two did not elicit similar responses. Immunization with HBsAg/P33d or y elicits antibody primarily focused on the p133-143 region of pre-S(2), which is crossreactive with native particles with only low reactivity with p156-174.
**TABLE 6**

Fine specificity of the antibody response to immunization with the synthetic pre-S(2) immunogen p133-174d.

<table>
<thead>
<tr>
<th>STRAIN*</th>
<th>TIME (WKS)</th>
<th>HBsAg/P33</th>
<th>ANTIBODY TITER (1/DILUTION)</th>
<th>p133-174d</th>
<th>p120-145d</th>
<th>p156-174</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>d</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>y</td>
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<tr>
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<td>10,240</td>
<td>640</td>
<td>163,840</td>
<td>640</td>
<td>20,480</td>
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<td></td>
<td>4</td>
<td>655,360</td>
<td>10,240</td>
<td>655,360</td>
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<td>163,840</td>
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<td>640</td>
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<td>81,920</td>
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<td>5,120</td>
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<td>B10.BR</td>
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<td>163,840</td>
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<td>1,280</td>
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<td>2,560</td>
<td>640</td>
<td>163,840</td>
<td>20,480</td>
<td>2,560</td>
</tr>
</tbody>
</table>

*Groups of 5 mice each of the indicated strains were immunized i.p. with 100 μg of p133-174d emulsified in CFA. Two and 4 weeks post-immunization sera was pooled and analyzed for IgG antibody reactive with the indicated antigens by ELISA. The antibody titer is expressed as the reciprocal dilution of serum which yielded 3x the O.D. reading of preimmunization sera.
Synthetic Immunogen p151-174(133-143)/

The effect of reversing the orientation of
the B and T cell sites was examined. A composite pre-
S(2)-specific peptide was synthesized by placing
the cell recognition sequence, p151-174, N-terminal
to the dominant B cell sites at p133-143. This
peptide was designated p151-174(133-143)/d. The fine
specificity of the antibodies elicited is shown in
Table 7. In all strains, primary immunization
resulted in high titres focused primarily on p133-143
(as detected on the p120-145 adhered to solid phase).
These antibodies were also highly crossreactive
against native HBsAg/P33 of both subtypes in all
The fine specificity, group specificity and
crossreactivity mimicked that of HBsAg/P33, and was
quite different from p133-174. Although the p133-174
sequence is more "native-like", the synthetic peptide
with the reverse orientation displayed more "native-
like" reactivity. Besides the orientation, the
spacing of the reactive sites was different between
the two immunogens. Twelve residues separate the B
and T sites in p133-174, and only four residues in
p151-174(133-143).
TABLE 7
Fine specificity of the antibody response to immunization with the synthetic pre-S(2) immunogen p151-174(133-143)/d.

<table>
<thead>
<tr>
<th>STRAIN*</th>
<th>TIME (WKS)</th>
<th>HBsAg/P33</th>
<th>ANTI BODY TITER (1/DILUTION)</th>
<th>p120-145</th>
<th>p156-174</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>d</td>
<td>y</td>
<td>d</td>
<td>y</td>
</tr>
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<td>1,280</td>
<td>40,960</td>
<td>40,960</td>
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<td>20,480</td>
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<td>163,840</td>
<td>163,840</td>
</tr>
<tr>
<td>B10.S</td>
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<td>5,120</td>
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</tr>
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<td>10,240</td>
<td>163,840</td>
<td>81,920</td>
</tr>
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<td>1,280</td>
<td>163,840</td>
<td>10,240</td>
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<td>20,480</td>
<td>10,240</td>
<td>327,680</td>
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<td>5,120</td>
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</tr>
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<td>10,240</td>
<td>81,920</td>
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<td>5,120</td>
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<td>0</td>
<td>0</td>
<td>40,960</td>
<td>1,280</td>
</tr>
</tbody>
</table>

* Groups of 5 mice each of the indicated strains were immunized i.p. with 100 µg of p151-174(133-143)/d in CFA, and IgG antibody specific to the indicated antigens was determined 2 and 4 weeks post-immunization.

Strain B10 was immunized with 141-174(133-143)/d to include the dominant T cell site for this strain (i.e., p149-157 (13).
Influence of p151-174(133-143) on T cell Fine Specificity

B10.S mice were immunized with synthetic immunogens of the d subtype, including: p133-174, p151-174(133-143), p148-174, p156-174 and p161-174, and T cell proliferation was examined. Figure 12 shows that p133-174-primed cells recognised the p156-170 site, but not the p161-174 site. Conversely, p151-174(133-143)-primed T cells preferentially recognized the p161-174 site. Immunization with the other peptides elicited equivalent responses to p156-170 and p161-174.

These results indicate that the larger context within which a T cell site exists may influence its immunogenicity. For example, the p161-174 sequence is not immunogenic in the context of p133-174, and yet represents the dominant T cell recognition site within the p151-174(133-143) immunogen. Also of interest is the fact that p161-174 was most effective in terms of priming native HBsAg/P33-specific T cell recognition.

Antibody Production by Immunogens of Varying Orientation

A summary of immunization results involving murine strains with disparate H-2 haplotypes and three pre-S(2) immunogens (d subtype) of different orientation is presented in Table 8. The p133-174 and p151-174(133-143) peptides combine the B cell site with the C-terminal T cell recognition region in different orientations, and the p120-145 peptide combines the B cell site with the N-terminal 120-132 sequence.
<table>
<thead>
<tr>
<th>STRAIN*</th>
<th>IMMUNOGEN</th>
<th>ANTIbody TITER (1/Dilution)</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>HBsAg/P33</td>
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<tr>
<td>B10</td>
<td>120-145(^b)</td>
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</tr>
<tr>
<td>B10.S</td>
<td>120-145</td>
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<tr>
<td></td>
<td>133-174</td>
<td>1,280</td>
</tr>
<tr>
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<td>151-174(133-143)</td>
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<tr>
<td>B10.M</td>
<td>120-145</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>133-174</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>151-174(133-143)</td>
<td>20,480</td>
</tr>
<tr>
<td>B10.BR</td>
<td>120-145</td>
<td>20,480</td>
</tr>
<tr>
<td></td>
<td>133-174</td>
<td>320</td>
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<td>20,480</td>
</tr>
<tr>
<td>B10.D2</td>
<td>120-145</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>133-174</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>151-174(133-1430)</td>
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<td>C57L</td>
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<td></td>
<td>151-174(133-143)</td>
<td>1,280</td>
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</tbody>
</table>

* Groups of 5 mice each of the strains shown were immunized i.p. with 100 μg in CFA of the indicated synthetic pre-S(2) immunogens. Sera were collected 4 weeks post-immunization and analyzed for IgG antibodies specific for the indicated antigens.

\(^b\) The boxes represent the location of the dominant B cell epitopes of the pre-S(2) region represented by residues 133-143.
Although pl20-145 elicited high titer anti-pl20-145 antibody crossreactive with HBsAg/P33 of both subtypes in C\(_5\)H\(_{\text{Q}}\) and B10.BR mice, and to a lesser extent in B10 mice, B10.S, B10.M and B10.DR\(_2\) strains were either nonresponders or low responders, suggesting a lack of recognition ability of the pl20-145 sequence.

pl33-174, elicited antibodies from all strains, indicating recognition capability of the C-terminal half of the pre-S(2) region. The anti-pl56-174 antibody produced is crossreactive with HBsAg/P33, however it is subtype-specific for both pl56-174 and HBsAg/P33. Immunization with pl51-174(133-143) [pl41-174(133-143) in the case of B10 mice] elicited high titer ant-pl33-143 reactive antibody and little anti-pl56-174 antibody. The anti-peptide antibodies are crossreactive against both subtypes of HBsAg/P33 in all strains except C\(_5\)H\(_{\text{Q}}\). Therefore, in most strains, pl51-174(133-143) was clearly the superior immunogen and most faithfully mimicked the HBsAg/P33-induced response.

**Discussion**

As predicted by the T cell recognition experiments, B10.M mice that do not produce antibody upon immunization with the ayw subtype of the pre-S(2) region, are capable of efficient anti-pre-S(2) antibody production after immunization with HBsAg/P33 of the adw\(_2\) subtype. This is the most dramatic example of the influence of subtype on antibody production, a pre-S(2) nonresponder strain is converted to a pre-S(2) responder strain simply by immunizing with the alternate subtype.

Because low-to-nonexistent responsiveness of human recipients of an HBsAg/P25 vaccine has recently
been correlated with certain MHC haplotypes, it is reasonable to assume that the human pre-S(2)-specific immune response is regulated by MHC-linked genes independently of S region regulation, as has been demonstrated in the mouse. Therefore, viral subtype influence is to be reasonably expected in T cell recognition of the pre-S(2) region in man.

Figure 13 represents a summary of experience with two synthetic pre-S(2) immunogens as compared to HBsAg/P33.

1. Immunization of B10.S mice with native HBsAg/P33 elicits antibody primarily specific for p133-139 (B₁) and to a lesser extent p137-143 (B₂). Two overlapping T cell recognition sites (T₁ and T₂) are also seen at p156-170 and p161-174 respectively.

2. The same mice immunized with p133-174, which is linearly similar to native synthetic pre-S(2) immunogen, produce antibodies to a unique site p156-174, and significantly less to B₁ and B₂. At the T cell level, primed T cells recognize the T₁ epitope exclusively, and the antibody produced is not highly crossreactive on native HBsAg/P33.

3. Immunization with p151-174(133-143), on the other hand, results in a more "native-like" antibody response, i.e., directed primarily at B₁ and B₂, and highly crossreactive with native HBsAg/P33. In contrast with p133-174, p151-174(133-143)-primed cells recognize T₂ preferentially.

Whatever cellular mechanisms may explain the enhanced immunogenecity of p151-174(133-143), this composite peptide represents an efficient synthetic pre-S(2) immunogen in the majority of MHC haplotypes
tested. Therefore, it is reasonable to expect that a similar orientation effect will obtain in man, and should be considered in any attempt to "engineer" a superior vaccine.

Although the present invention has now been described in terms of certain preferred embodiments, and exemplified with respect thereto, one skilled in the art will readily appreciate that various modifications, changes, omissions and substitutions may be made without departing from the spirit thereof. It is intended, therefore, that the present invention be limited solely by the scope of the following claims.
WHAT IS CLAIMED IS:

1. A pre-S(2) T cell epitope polypeptide of 16 to 50 amino acid residues consisting essentially of a first amino acid residue sequence corresponding to a formula selected from the group consisting of:
   residues 136-155, 136-174,
   148-159, 148-174, 149-165, 152-159,
   154-170, 156-165, 156-167, 156-170,
   159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/\( \beta \); and
   a second amino acid residue sequence corresponding to a formula selected from the group consisting of:
   residues 136-155, 136-174,
   146-165, 148-155, 148-165, 148-174,
   151-165, 151-170, 156-165, 159-169,
   and 161-174 of the pre-S(2) region of HBsAg/\( \gamma \);
2. A composite polypeptide comprising a first polypeptide (a) operatively linked to the amino terminus of a second polypeptide (b) wherein:
   polypeptide (a) consists essentially of a pre-S(2) T cell epitope polypeptide of 6 to 50 amino acid residues including an amino acid residue sequence that corresponds to at least one amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 148-159, 148-174, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/\( \beta \) and residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/\( \gamma \); and
   polypeptide (b) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence of HBsAg that contains a native B
cell epitope.

3. The composite polypeptide of claim 2
wherein said B cell epitope is a group-specific B cell epitope.

4. The composite polypeptide of claim 2
wherein polypeptide (b) comprises an amino acid
residue sequence that corresponds to subtype-specific
B cell epitope.

5. The composite polypeptide of claim 2
wherein polypeptide (b) comprises an amino acid
residue sequence that corresponds to an S region B
cell epitope.

6. The composite polypeptide of claim 2
wherein polypeptide (b) comprises an amino acid
residue sequence that corresponds to a pre-S(1) region
B cell epitope or a pre-S(2) region B cell epitope.

7. The composite polypeptide of claim 2
wherein polypeptide (a) consists of a pre-S(2) T cell
epitope polypeptide that corresponds to residues 148-
174 of the pre-S region of HBsAg/d or HBsAg/y.

8. A composite polypeptide of at least 14
and not more than 100 amino acid residues comprising a
first polypeptide (a) linked to the amino terminus of
a second polypeptide (b) wherein:

    polypeptide (a) comprises a pre-S(2) T
cell epitope having an amino acid residue sequence
that corresponds to at least one amino acid residue
sequence selected from the group consisting of
residues 136-155, 136-174, 148-159, 148-174, 149-165,
152-159, 154-170, 156-165, 156-167, 156-170, 159-167,
159-169 and 159-174 of the pre-S(2) region of HBsAg/d
and residues 136-155, 136-174, 146-165, 148-155,
148-165, 148-174, 151-165, 151-170, 156-165, 159-169,
and 161-174 of the pre-S(2) region of HBsAg/y; and
polypeptide (b) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence of HBsAg that contains a native B cell epitope.

9. The composite polypeptide of claim 8 wherein said B cell epitope is a group-specific epitope.

10. The composite polypeptide of claim 8 wherein polypeptide (b) comprises an amino acid residue sequence that corresponds to a native pre-S(2) region B cell epitope.

11. The composite polypeptide of claim 10 wherein polypeptide (b) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence of the pre-S(2) region of HBsAg selected from the group consisting of:

residues 133-139, 133-143, and 137-143.

12. A composite polypeptide of claim 11 comprising an amino acid residue sequence corresponding to at least one amino acid residue sequence selected from the group consisting of residues (141-174)-(133-140), (146-160)-(133-143), (148-174)-(133-143), (151-165)-(133-143), (151-165)-(133-143) and (151-174)-(133-143) of the pre-S(2) region of HBsAg.

13. A composite subtype T cell hepatitis B virus (HBV) vaccine, said vaccine comprising polypeptides (a1) and (a2) wherein:

polypeptide (a1) comprises an amino acid sequence corresponding to the amino acid residue sequence of a native HBsAg/α T cell epitope; and

polypeptide (a2) comprises an amino acid residue sequence corresponding to the amino acid residue sequence of a native HBsAg/γ T cell epitope.
14. The vaccine of claim 13 wherein at least one of said polypeptides corresponds to a native press region T cell epitope.

15. The vaccine of claim 14 wherein polypeptide (a1) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 148-159, 148-174, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/\d.

16. The vaccine of claim 14 wherein polypeptide (a2) comprises an amino acid residue sequence that corresponds to an amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/\y.

17. The vaccine of claim 13 additionally comprising at least one of polypeptide (b1) and polypeptide (b2) which is operatively linked to at least one of polypeptide (a1) and polypeptide (a2), wherein polypeptides (b1) and (b2) each comprise an amino acid residue sequence corresponding to the amino acid residue sequence of a native HBsAg native B cell epitope.

18. The vaccine of claim 17 wherein polypeptides (a1) and (a2) are operatively linked to polypeptides (b1) and (b2), respectively.

19. The vaccine of claim 18 wherein polypeptides (b1) and (b2) have the same amino acid sequence.

20. The vaccine of claim 18 wherein polypeptides (a1) and (a2) are operatively linked to polypeptide (b1).
21. The vaccine of claim 13 wherein said native B cell epitope is a group-specific B cell epitope.

22. The vaccine of claim 13 wherein said native T cell epitope is a HBsAg pre-S T cell epitope.

23. The vaccine of claim 13 wherein said native B cell epitope is a HBsAg pre-S B cell epitope.

24. The vaccine of claim 13 wherein polypeptide (a1) is operatively linked to polypeptide (b1) and comprises HBsAg/p39ad.

25. The vaccine of claim 13 wherein polypeptide (a2) is operatively linked to polypeptide (b2) and comprises HBsAg/p33av.

26. A vaccine of claim 13 comprising HBsAg/p39ad and HBsAg/p39av.

27. The vaccine of claim 13 wherein at least one of said polypeptides (a1) and (a2) is present in a particle.

28. The vaccine of claim 13 wherein both of said polypeptides form a heterogeneous particle.

29. A method of mitigating nonresponsiveness to an HBV vaccine comprising administering, either prior to or together with administration of said vaccine, an effective amount of a composite subtype T cell immunogen comprising at least one native HBsAg/\d pre-S T cell epitope and at least one native HBsAg/\y pre-S T cell epitope.

30. The method of claim 29 wherein said immunogen comprises:

an amino acid residue sequence that corresponds to an amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 148-159, 148-174, 149-165, 152-159, 154-170, 156-165, 156-167, 156-170, 159-167, 159-169 and 159-174 of the pre-S(2) region of HBsAg/\d; and
an amino acid residue sequence that corresponds to an amino acid residue sequence selected from the group consisting of residues 136-155, 136-174, 146-165, 148-155, 148-165, 148-174, 151-165, 151-170, 156-165, 159-169, and 161-174 of the pre-S(2) region of HBsAg/y.

31. The method of claim 30 wherein said immunogen and said vaccine are administered simultaneously as a composition that comprises HBsAg/p39ad and HBsAg/p39ay.

32. A synthetic carrier moiety comprising a sequence corresponding to the HBsAg/d or /y pre-S(2) sequence, 148-174, operatively linked to one or more polypeptide immunogen.

33. The carrier moiety described in claim 32 wherein said carrier is linked to the N-terminal of said polypeptide immunogen.

34. A vaccine comprising the two major subtypes ay and ad of each of a plurality of epitopes in any permutation or combination thereof.

35. A vaccine as described in claim 34 wherein said epitopes are on separate molecules linked to an insoluble structure.
FIG. 2
FIG. 12

FIG. 13

SUBSTITUTE SHEET
### INTERNATIONAL SEARCH REPORT

**International Application No:** PCT/US91/03268

**I. CLASSIFICATION OF SUBJECT MATTER**

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/12, 39/00, 39/02; C07K 3/00

U.S. CL.: 424/89, 88; 530/324, 403

**II. FIELDS SEARCHED**

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Documentation searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched.

### III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of Document</th>
<th>Relevant to Claim No</th>
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<tbody>
<tr>
<td>Y</td>
<td>US, A, 4,847,080 (MEURATH ET AL.) 11 July 1989, see the entire document.</td>
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<td>The Journal of Immunology, Volume 144, No. 9, issued 01 May 1990, MILICH ET AL., &quot;Importance Of Subtype In The Immune Response To The Pre-S(2) Region Of The Hepatitis B Surface Antigen&quot; I. T Cell Fine Specificity&quot;, pages 3535-3543, see the entire document.</td>
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**O** document referring to an oral disclosure, e.g. exhibition or other means.

**P** document published prior to the international filing date but later than the priority data claimed.

### IV. CERTIFICATION

**Date of the Actual Completion of the International Search:** 13 August 1991

**Date of Mailing of this International Search Report:** 12 SEP 1991

**International Searching Authority:**

ISA/US

**Abdel A. Mohamed**

Abdel A. Mohamed (vsh)

Form PCT/ISA/20 (second sheet) (January 1985)
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