

STRATEGICALLY MODIFIED HEPATITIS B CORE
PROTEINS AND THEIR DERIVATIVESDescription

5

Technical Field

The present invention relates to the intersection of the fields of immunology and protein engineering, and particularly to carrier proteins, and more particularly to a hepadnavirus nucleocapsid protein strategically modified with an inserted chemically-reactive amino acid residue, a pendently-linked hapten conjugate of that hepadnavirus nucleocapsid protein and to an immunogenic particle comprised of those conjugates.

15

Background of the Invention

It is known that antibodies can be raised to a small molecule by using a large immunogenic protein molecule as a carrier. The small molecule that derives enhanced immunogenicity by being conjugated to the carrier is called a hapten. The phenomenon of a relatively large molecule potentiating the immunogenicity of a small molecular entity to which it is attached is known in the art as the "carrier effect".

20

25

The portion of an immunogen recognized by the helper T cell (Th cell) is the T cell determinant or epitope. The portion of an immunogen that is bound by antibody is the B cell determinant or epitope. Carrier effects can be defined as immunity to one determinant, the "helper" or T (T_h) cell

30



-2-

determinant, of a multideterminant immunogen enhancing the immune response to another determinant, the B cell determinant.

It is now well established that most
5 immunogens require T cell help to induce B cells to produce antibodies. Thus, T_h cells, by recognizing helper determinants on the immunogen help B cells to make antibody against the immunogen.

The antigenic determinants recognized by T
10 helper cells (T_h) and B cells must be associated to form a single molecular entity, but they do not have to be covalently linked. See, Russel et al., *Nature*, 280:147 (1979), Lamb et al., *J. Immunol.*, 129:1465 (1982), Scherle et al., *J. Exp. Med.*, 164:1114 (1986)
15 and Lake et al., *Cold Spring Harbor Symp. Quant. Biol.*, 41:589 (1976). Some immunogens do not require T cell help to induce antibody formation, these are T-independent antigens.

A pathogen-related protein can be
20 immunologically mimicked by the production of a synthetic polypeptide whose sequence corresponds to that of a determinant of the pathogen. Such polypeptide immunogens are reported by Sutcliffe et al., *Nature*, 287:801 (1980), and Lerner et al., *Proc. Natl. Acad. Sci. USA*, 78:3403 (1981).
25

Gerin et al., *Proc. Natl. Acad. Sci. USA*, 80:2365 (1983), showed limited protection of chimpanzees from hepatitis B virus upon immunization with carrier-bound synthetic polypeptides having
30 amino acid residue sequences that correspond to the sequence of a determinant portion of HBsAg; in

-3-

particular, residues 110-137 of the "S" (surface) region. However, the carrier protein used in those studies was keyhole limpet hemocyanin (KLH), a T cell-dependent carrier that is not fit for use in medical applications to humans because it is a source of irritation that leads to severe inflammation.

T cell-stimulating carrier proteins capable of enhancing the immunogenicity of haptens that do not produce unacceptable side effects in human subjects are often immunogenic natural proteins. For example, tetanus toxoid (TT) has been frequently used when a carrier suitable for human administration was needed. However, the use of tetanus toxoid as a carrier was restricted due to problems with dosage limitations and risk of sensitization to the toxoid itself. In addition, an epitope-specific suppression of response to the carried hapten can occur in individuals already immunized against tetanus.

The hepatitis B surface protein has been proposed as a carrier for heterologous epitopes. Delpeyroux et al., *Science*, 233:472-475 (1986), reported the use of the HBV surface protein (S protein) as a carrier for a poliovirus polypeptide hapten. Those investigators constructed a recombinant deoxyribonucleic acid (DNA) protein expression vehicle that produces a fusion protein, designated HBsPolioAg, capable of forming particles closely resembling authentic 22-nanometer HBsAg particles. HBsPolioAg consists of HBV S protein having an 11 amino acid residue sequence insert

-4-

corresponding to amino acids 93-103 of capsid protein VPI of poliovirus type 1 (Mahoney strain).

Hepadnavirus nucleocapsid proteins have been used as hapten carriers. Heterologous immunogenic peptide sequences inserted internally in the hepatitis B core, expressed as fusion particles, elicited very high immune responses in immunized animals in the absence of adjuvants. B.E. Clarke et al. *Vaccines* 91:313-318 (1991); F. Schödel et al. *J. Virol.* 66(1):106-114 (1992). U.S. Patent Nos. 4,818,527, 4,882,145, and 5,143,726, the disclosures of which are incorporated herein by reference, describe the use of the carrier effect with hepatitis B virus nucleocapsid protein to enhance the immunogenicity of an operatively linked polypeptide hapten. Those patents describe the linking of a polypeptide hapten to hepatitis B virus nucleocapsid protein through an amino acid residue side chain that occurs naturally in the hepatitis B nucleocapsid protein sequence.

Hepadnavirus nucleocapsid proteins are fairly well studied. SEQ ID NOS:1 and 2 are the DNA and amino acid sequences of the human hepatitis B core protein (HBc), subtype ayw, as described in U.S. Patent Nos. 4,818,527, 4,882,145, and 5,143,726. Other hepadnavirus nucleocapsid protein sequences are also known in the art, see e.g. SEQ ID NOS: 3-13.

There are reasons to select hepadnavirus nucleocapsid proteins as a carrier over other carriers used in the art, such as keyhole limpet hemocyanin (KLH), BCG, tetanus toxoid and diphtheria

-5-

toxoid. KLH, BCG, tetanus toxoid and diphtheria
toxoid are non-particulate, whereas hepadnavirus
nucleocapsid proteins tend to aggregate into
"particles". HBc particles tend to have a higher
5 immunogenicity than hepatitis B surface antigen
(HBsAg) particles. D.R. Milich et al., *Science*,
234:1398-1401 (1986). HBc is both a T cell-
independent and a T cell-dependent immunogen. *Id.*
HBc is one of the most immunogenic proteins known.
10 Almost all hepatitis B-infected people develop a
powerful immune response to core. J.H. Hoofnagle,
Semin. Liver Dis., 1(1):7-14 (1981). HBc can provide
universal responsiveness, irrespective of genetic
background. *Id.* HBc directly activates T cells.
15 HBc elicits strong T_h cell responses. HBc is
efficiently processed and presented by antigen-
presenting cells. Due to the inherently high
immunogenicity of HBc, complex adjuvants are
typically not required, for example, the common and
20 inexpensive alum is sufficient.

The family *hepadnaviridae* is a family of
enveloped animal viruses with a core of DNA that
cause hepatitis B in humans. The *hepadnaviridae* are
not responsible for human hepatitis A (a single-
25 stranded RNA enterovirus), human hepatitis C
(*Flaviridae* family of single stranded RNA virus), or
human hepatitis D (a closed circular negative-sense
RNA satellite virus, "delta virus", that requires
hepatitis B virus for replication). The
30 *hepadnaviridae* family includes hepatitis viruses of
other species, e.g. woodchuck, duck, ground squirrel,

and heron, in addition to human and simian hepatitis B. Hepatitis B (HB) used hereinafter refers to the family *hepadnaviridae*, unless the discussion is referring to a specific example.

5 Hepatitis B core protein monomers self-assemble into stable aggregates known as hepatitis B core protein particles (HBc particles). For example, human HBc particles are 27 nanometers (nm) in diameter. Conway et al., *Nature*, **386**:91-94 (1997),
10 describe the structure of human HBc particles at 9 Ångstrom resolution, as determined from cryo-electron micrographs. Bottcher et al., *Nature*, **386**:88-91 (1997), describe the polypeptide fold for the human HBc monomers, and provide an approximate numbering
15 scheme for the amino acid residues at which alpha helical regions and their linking loop regions form. Bottcher et al. propose a loop from about residues 78 to 82 of the hepatitis B core protein.

 Using synthetic peptides and monoclonal
20 antibodies, the immunodominant loop region of HBc was mapped to about amino acid residues 75 to 83. G. Colucci et al., *J. Immunol.*, **141**:4376-4380 (1988). Two immunodominant linear epitopes were reported by other workers at amino acid residues 75 to 85 and 130
25 to 140. Salfeld et al. *J. Virol.* **63**:798 (1989).

 Insertion mutants of the hepatitis B core protein still are able to form core particles when foreign epitopes are cloned into the immunodominant loop region of HBc. P. Pumpens et al.,
30 *Intervirology*, **38**:63-74 (1995). The HBc fusion

8 AUG 2002

-7-

proteins form particles in prokaryotic and eukaryotic expression systems. *Id.*

The ability to use a protein as a carrier for a pendently-linked hapten depends upon several factors that have been studied with respect to HBc. Chemically-reactive amino acid side chains, such as lysine (K), aspartic acid (D), glutamic acid (E), and reduced cysteine residues (C), provide functional groups that can be useful for modifying polypeptides.

The hepatitis B core protein sequence has several chemically-reactive amino acid side chains in the native sequence. Core has three primary amino groups, one at the amino terminus, and two lysine residues (K5 and K96), along with four cysteine residues (C48, C61, C107 and C183). There are several carboxylic acid groups, D (2, 4, 22, 29, 32, 78, 83) and E (8, 14, 40, 43, 46, 64, 77, 113, 117, 145, 179) and the carboxy terminus.

However, the native, unmodified hepatitis B core protein particle does not exhibit appreciable chemical reactivity of the amino acid side chains in the native sequence. The chemical reactivity of an amino acid side chain in a protein depends upon the nature of the amino acid side chain, and its environment in the folded protein.

As is discussed in detail hereinafter, it has now been found that the problem of low reactivity of the amino acid side chains in native hepatitis B nucleocapsid protein can be overcome by inserting a chemically-reactive amino acid side chain into the HBc protein sequence. A strategically modified

hepadnavirus core protein particle of the present invention exhibits substantially enhanced reactivity toward derivatization of HBc particles with chemically linked haptens, and provides enhanced immunogenicity to those linked haptens.

These modified HBc proteins and their pendently-linked hapten conjugate derivatives are discussed in the disclosure that follows.

Brief Summary of the Invention

The present invention relates to a strategically modified hepatitis B core (HBc) protein that is linked to a pendent hapten through chemically-reactive amino acid residue inserted into the HBc sequence. The contemplated strategic modification of HBc is an insert mutation of the HBc protein. A contemplated insert is 1 to about 40 amino acid residues in length, preferably 1 to 10 amino acid residues in length, and includes a chemically-reactive amino acid residue.

The insert is provided to the region corresponding to amino acid residues about 50 to about 100 of the hepatitis B core protein sequence shown in SEQ ID NO:2. The preferred region of insertion corresponds to the hepatitis B core protein immunodominant loop region at about amino acid residue 70 to about 90, more preferably the loop tip region at about amino acid 78 to about 82 and most preferably at amino acid 78 to amino acid 80.

Such an introduced chemically-reactive amino acid residue is characterized in that it has a

-9-

chemically-reactive side chain that provides a chemical group for pendently linking the strategically modified HBc to the hapten. Typically, the chemically-reactive amino acid residue is a lysine, cysteine, or histidine residue or a carboxyl-containing residue such as aspartic acid or glutamic acid, preferably lysine or a carboxyl-containing residue, and most preferably lysine.

The hapten bonded to the chemically-reactive amino acid residue is any compound of interest for generating an immune response, and is typically a B cell determinant. Preferably, the hapten is a polypeptide hapten, a carbohydrate hapten, or a non-peptidal/non-saccharidal (chemical) hapten. In one embodiment of the invention, the hapten is a pathogen-related hapten, such as a B cell determinant of a pathogen.

In another embodiment of a strategically modified hepatitis B core protein conjugate, the strategically modified hepatitis B core protein also has a T cell stimulating amino acid residue sequence operatively linked to the carboxy terminus of the hepatitis B core amino acid sequence. Preferably, both the hapten and the T cell stimulating amino acid residue sequence are pathogen-related, most preferably, both are related to (from) the same pathogen.

In the above embodiment of the invention, the response to a B cell epitope is boosted by also providing the T helper (T_h) cell determinant. In this preferred embodiment of the invention, such a T_h cell

-10-

determinant is from the same pathogen as the B cell determinant hapten that is pendently linked to the strategically modified hepatitis B core protein in order to provide pathogen-specific T cell memory in addition to the hepatitis B core protein antigen-specific T cell memory.

A strategically modified hepatitis B core protein conjugate contains a hapten that is pendently linked to a strategically modified hepatitis B core protein. Looked at differently, a before-described strategically modified hepatitis B core protein can be considered to have three peptide-linked domains, I, II and III (numbered consecutively from the amino terminus). Domain I comprises a sequence that corresponds to residues numbered about 10 to 50 of the amino acid sequence of hepatitis B core protein of SEQ ID NO:2, and preferably corresponds to residues numbered 1 to 50 of that sequence. Domain II is bonded to the carboxy terminal residue of Domain I. Domain II corresponds to residues numbered 50 to 100 of the amino acid sequence of hepatitis B core protein of SEQ ID NO:2. Domain III comprises a sequence that is bonded to the carboxy terminal residue of Domain II. Domain III corresponds to residues numbered 100 to about 140 of the amino acid sequence of hepatitis B core protein, and preferably corresponds to residues numbered 100 to about 149 of that sequence.

In an embodiment of the invention discussed before, a strategically modified hepatitis B core protein additionally has a Domain IV exogenous to HBc

that is peptide-bonded to the carboxy terminal residue of Domain III to provide a fusion protein. Domain IV is a T cell epitope.

A strategically modified hepatitis B core protein particle of the invention is made of assembled hepatitis B core protein where a plurality of the subunits are strategically modified hepatitis B core protein subunits. Also contemplated is a particle comprised of a mixture of strategically modified hepatitis B core protein subunits and other hepatitis B core protein subunits.

A contemplated strategically modified hepatitis B core protein particle conjugate is comprised of assembled hepatitis B core protein subunits where a plurality of the subunits are strategically modified hepatitis B core protein subunits. In this embodiment, a hapten is pendently linked to a hepatitis B core protein subunit. Preferably, the hapten is pathogen-related. As above, a T cell stimulating amino acid residue sequence can be peptide-bonded to the carboxy terminal residue of the sequence corresponding to hepatitis B core protein. Preferably that pathogen-related T cell determinant is related to the same pathogen as the pathogen-related hapten.

A strategically modified hepatitis B core protein particle conjugate of the invention has pendently-linked hapten. In a contemplated embodiment of the particle conjugate, the particle is made up of a mixture of strategically modified hepatitis B core protein subunits having pendently-

-12-

linked haptens, and other hepatitis B core protein subunits. In one embodiment, about 0.1 to about 0.5 of the strategically modified hepatitis B core protein subunits are pendently linked to a hapten.

5 Also contemplated is a particle conjugate that is made up of a mixture of strategically modified hepatitis B core protein subunits and other hepatitis B core protein subunits.

A before-described strategically modified hepatitis B core protein of the invention includes a peptide insert containing a chemically-reactive amino acid residue. That insert can be, but is typically not itself a separate B cell antigenic determinant, although some B cell immunogenicity of the insert can

10 be exhibited merely because of the placement of the insert into the HBc protein or particle. Such an insert can be and in some embodiments is a T cell epitope. Placement of an insert into the HBc loop region greatly diminishes the HBc immunogenicity and

15 antigenicity of the resulting molecule.

20

An inoculum of the invention comprises an immunogenic amount of the strategically modified hepatitis B core protein conjugate of the invention. When the pendently-linked hapten is a pathogen-

25 related hapten, the inoculum can be used as a vaccine to protect a mammal treated with the inoculum from that pathogen. Thus, in one embodiment of the invention, a strategically modified hepatitis B core protein conjugate is used as a vaccine to provide

30 protection against the pathogen from which the hapten is derived. More preferably, the inoculum is

comprised of strategically modified hepatitis B core protein particle conjugate as the immunogen.

The present invention has several benefits and advantages.

5 One benefit of a contemplated modified HBc protein is that the protein can be derivitized while in the aggregated form of HBc particles.

An advantage of the invention is that the modified HBc protein displays appreciably enhanced
10 chemical reactivity toward derivatization, as compared to use of the N-terminal primary amine, for example.

Another benefit of a contemplated modified HBc protein is that the chemistry of derivatization
15 of such side chains is well-studied, straightforward and relatively predictable.

Another advantage of a contemplated modified HBc protein is that it enhances the immunologic response to the conjugated hapten with
20 which it is derivitized.

Yet another benefit of a contemplated modified HBc protein is that it is unlikely to produce undesirable immunologic side effects in humans.

25 Still further benefits and advantages of the invention will be apparent to the skilled worker from the discussion that follows.

Brief Description of the Drawings

30 In the figure forming a portion of this disclosure Scheme 1 illustrates a reaction sequence

-14-

for pendently linking a hapten to a strategically modified hepatitis B core protein (sm-HBc) particle using sulpho-succinimidyl 4-(N-maleimidomethyl) cyclohexane 1-carboxylate (sulpho-SMCC). The sm-HBc
5 particle is depicted as a box having (for purposes of clarity of the figure) a single pendent amino group.

Detailed Description of the Invention

10

A. Definitions

The term "antibody" refers to a molecule that is a member of a family of glycosylated proteins called immunoglobulins, which can specifically
15 combine with an antigen.

The word "antigen" has been used historically to designate an entity that is bound by an antibody, and also to designate the entity that induces the production of the antibody. More current
20 usage limits the meaning of antigen to that entity bound by an antibody, whereas the word "immunogen" is used for the entity that induces antibody production. Where an entity discussed herein is both immunogenic and antigenic, reference to it as either an immunogen
25 or antigen will typically be made according to its intended utility.

The word "hapten" is used to describe molecules that are capable of stimulating an immune response (e.g., production of antibody) when
30 chemically coupled to a protein carrier. The word is often used for small nonantigenic molecules in the

- 8 AUG 2002

art, but herein, it merely refers to the molecule that is to be pendently linked to the carrier protein, even if it is antigenic or not small.

"Antigenic determinant" refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site or T cell receptor. The term is also used interchangeably with "epitope".

The noun "conjugate" as used herein refers to a molecule formed from a hapten pendently linked through an amino acid residue side chain to a carrier.

The term "conservative substitution" as used herein denotes that one amino acid residue has been 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 antibodies raised to such a polypeptide also immunoreact with the corresponding polypeptide having the unsubstituted amino acid.

The term "corresponds" in its various grammatical forms as used in relation to peptide sequences means the peptide sequence described plus

-16-

or minus up to three amino acid residues at either or both of the amino- and carboxy-termini and containing only conservative substitutions in particular amino acid residues along the polypeptide sequence.

5 "Epitope" refers to that portion of a molecule that is specifically bound by a T cell antigen receptor or an antibody combining site. "Epitope" and "determinant" are used interchangeably.

 As used herein, the term "fusion protein" designates at least two amino acid residue sequences not normally found linked together in nature operatively linked together end-to-end (head-to-tail) by a peptide bond between their respective terminal amino acid residues.

15 The phrase "hepatitis B" as used here refers in its broadest context to any member of the family *hepadnaviridae*, a family of enveloped DNA-containing animal viruses that can cause hepatitis B in human.

20 The phrase "HBc" as used here refers to T cell stimulating proteins having an amino acid residue sequence that corresponds to an amino acid residue sequence encoded by the hepatitis B virus (HBV) nucleocapsid protein gene. An exemplary well-known naturally occurring protein encoded by the human HBV nucleocapsid gene is the "core" protein, subtype ayw, having the biological sequences of SEQ ID NOs: 1 and 2. Galibert, et al., *Nature* **281**:646 (1979). HBeAg protein, the precursor to HBc, includes the sequence of the hepatitis B core protein and a "pre-core" sequence at the amino terminus

25

30

-17-

thereof, as shown in SEQ ID NOs: 8 and 9 in the case of a ground squirrel hepatitis B nucleocapsid gene. The core protein sequence begins at amino acid position 31 therein, thus corresponding to amino acid residue number 1 of SEQ ID NO:2. The sequences for other hepatitis B core proteins are known in the art. Human hepatitis B virus core protein subtype adr is provided in SEQ ID NOs: 3 and 4, and subtype adw is provided in SEQ ID NOs: 5 and 6. Ono et al., *Nucl. Acids Res.* 11:1747 (1983). Sequences are also provided for woodchuck hepatitis B core protein at SEQ ID NO:7 [Schödel et al., *Adv. Viral Oncol.* 8:73-102 (1989)], ground squirrel at SEQ ID NOs:8 and 9, heron at SEQ ID NOs:10 and 11, and duck at SEQ ID NOs:12 and 13. For clarity, the amino acid numbering system shown in SEQ ID NOs:1 and 2 with respect to human hepatitis B core protein subtype ayw is used as a benchmark herein. Other HBc sequences can be aligned with that sequence using standard biological sequence alignment programs and protocols to determine the amino acid residues that "correspond to the hepatitis B core protein sequence of SEQ ID NO:2", see e.g. Schödel et al., *Adv. Viral Oncol.* 8:73-102 (1989).

25 If reference is made to a polypeptide portion of any of the above described naturally occurring HBV nucleocapsid gene encoded proteins, that reference is explicit, either by stating, for example, that a T cell stimulating portion of the particular protein is referred to or by explicitly designating the particular portion of the sequence,

-18-

as by indication of the included amino acid residue positions.

The term "immunoreact" in its various forms means specific binding between an antigen as a ligand and a molecule containing an antibody combining site such as a Fab portion of a whole antibody.

The phrase "operatively linked" as used herein means that the linkage does not interfere with the ability of either of the linked groups to function as described; e.g., to function as a T or B cell determinant.

The phrase "pendently linked" refers to a single linkage, either direct or via a bridge, from a HBc protein to another molecule at other than the amino or carboxy termini. The phrase is used herein to describe the linkage between a hapten and a chemically-reactive amino acid side chain of a strategically modified hepatitis B core protein.

"Macromolecular assembly" refers to a non-covalently bonded aggregate of protein subunits. Typically in this invention, the protein subunit is a strategically modified hepatitis B core protein monomer. As described in more detail hereinafter, those core protein monomers usually self-assemble into spherical "core particles" having either 90 or 120 core protein dimers (a total of 180 or 240 core protein subunits). A spherical core particle is an example of a macromolecular assembly.

The phrase "pathogen-related" as used herein designates a B cell or T cell immunogen that is capable of inducing the production of antibodies

-19-

that immunoreact with a pathogen in native form.
Exemplary pathogen-related B cell and T cell
immunogens are illustrated hereinafter.

The words "polypeptide" and "peptide" as
5 used interchangeably throughout the specification and
designate a linear series of amino acid residues
connected one to the other by peptide bonds between
the alpha-amino and carboxy groups of adjacent amino
acids. Polypeptides can be variety of lengths,
10 either in their neutral (uncharged) forms or in forms
which are salts, and either free of modifications
such as glycosylation, side chain oxidation, or
phosphorylation or containing these modifications.
It is well understood in the art that amino acid
15 residue sequences contain acidic and basic groups,
and that the particular ionization state exhibited by
the peptide is dependent on the pH of the surrounding
medium when the protein is in solution, or that of
the medium from which it was obtained if the protein
20 is in solid form. Also included in the definition
are proteins modified by additional substituents
attached to the amino acid side chains, such as
glycosyl units, lipids, or inorganic ions such as
phosphates, as well as modifications relating to
25 chemical conversions of the chains, such as oxidation
of sulfhydryl groups. Thus, "polypeptide" or its
equivalent terms is intended to include the
appropriate amino acid residue sequence referenced,
subject to those of the foregoing modifications which
30 do not destroy its functionality. A peptide or
polypeptide used as a hapten typically contains fewer

-20-

than 70 amino acid residues, and more typically contains a linear chain of about 5 to about 40 amino acid residues, and more preferably about 10 to about 25 residues. It is noted that a contemplated
5 polypeptide hapten can be longer than 70 residues, but such a polypeptide is shorter than the naturally occurring protein that shares its sequence.

The word "protein" designates a polypeptide having about 70 or more amino acid residues, and is a
10 naturally occurring entity.

The words "secrete" and "produce" are often used interchangeably in the art as to cells from which antibody molecules are obtained. Cells that produce antibodies may, however, not secrete those
15 molecules into their environment. Herein, the antibody molecules are secreted and are obtained from the blood stream (humoral antibody). Nevertheless, antibodies are generally referred to as being "produced" in keeping with the phrase utilized in the
20 art.

All amino acid residues identified herein are in the natural or L-configuration. In keeping with standard polypeptide nomenclature, [*J. Biol. Chem.*, 243, 3557-59 (1969)], abbreviations for amino
25 acid residues are as shown in the following Table of Correspondence, Table 1.

-21-

Table 1.

TABLE OF CORRESPONDENCE

<u>SYMBOL</u>		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
Z	Glx	L-glutamic acid or L-glutamine
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
B	Asx	L-aspartic acid or L-asparagine
C	Cys	L-cysteine

5 B. Strategically Modified Hepatitis B Core Protein

The present invention contemplates a
 strategically modified *hepadnaviridae* core ("HBc")
 protein that has an inserted chemically reactive
 amino acid residue for pendently linking with haptens
 10 such as polypeptides and carbohydrates. The
 strategic modification of the invention is the

-22-

insertion of 1 to about 40 amino acid residues including a chemically-reactive amino acid residue into the hepatitis B core protein sequence in the region corresponding to amino acid residues 50 to 100 of the HBc sequence of SEQ ID NO:2. Such an introduced chemically-reactive amino acid residue has a side-chain that provides a functional group for pendently linking a hapten to the strategically modified carrier.

10 *Hepadnaviridae* have a nucleocapsid, or core, surrounded by a lipid envelope containing surface proteins. The nucleocapsid is a generally spherical aggregate of core proteins ("core antigen", HBcAg) dimers. In vitro, the hepatitis B core protein self-assembles into "particles", spherical shells of icosahedral symmetry made up of 90 or 120 hepatitis B core protein dimers, thus 180 or 240 protein subunits. The particles are about 280 or 310 Ångstroms in diameter, respectively. B. Bottcher et al., *Nature*, 386:88-91 (1997); J.F. Conway et al. *Nature* 386:91-94 (1997).

25 A contemplated strategically modified hepatitis B core protein also forms a macromolecular assembly. Such a particle can be present in the form of 180 or 240 protein subunits, although it does not have to be such a 90 or 120 dimer.

30 Hybrid core proteins with exogenous amino acid residues inserted in the region near amino acid residue 80 are reported to assemble into regular shells, even with inserts as large as 46 amino acids in length. A.I. Brown et al., *Vaccine*, 9:595-601

-23-

(1991); F. Schödel et al., *J. Virol.*, 66:106-114
(1992).

The *hepadnaviridae* core protein sequence used as a benchmark sequence herein is that of the human hepatitis B core protein, subtype ayw, shown in SEQ ID NOS:1 and 2. Other subtypes of the human hepatitis B virus are known. SEQ ID Nos: 3 and 4 are human HBc, subtype adr, and SEQ ID NOS:5 and 6 are HBc subtype adw. The sequences of various animal hepatitis core proteins are also published. The biological sequence of duck hepatitis core protein is disclosed herein as SEQ ID NO:12 and 13; a portion of the ground squirrel hepatitis nucleocapsid gene is at SEQ ID NO:8 and 9; woodchuck hepatitis core is at SEQ ID NO:7 and heron hepatitis core at SEQ ID NOS:10 and 11. Exemplary animal hepatitis B core proteins are aligned with human hepatitis B core protein by F. Schödel et al., *Adv. Viral Oncology* 8:73-102 (1989).

20 i. Strategic Modification of the Core Protein

The present invention contemplates a strategically modified hepatitis B core protein conjugate that comprises a hapten that is pendently linked to a strategically modified hepatitis B core protein (HBc). The strategically modified hepatitis B core protein itself comprises an amino acid sequence corresponding to the hepatitis B core protein amino acid sequence of SEQ ID NO:2 including the amino acid residues numbered about 10 to about 140 of that sequence. That HBc amino acid residue sequence additionally contains an exogenous amino

-24-

acid residue insert in the region corresponding to amino acid residues numbered about 50 to about 100 from the HBc amino terminus, wherein the exogenous insert (i) is 1 to about 40 amino acid residues in length, and (ii) contains a chemically-reactive amino acid residue. The hapten is pendently linked to the strategically modified HBc protein by means of a chemically-reactive amino acid residue present in the insert.

10 It is preferred that residues 1 through 10 of SEQ ID NO:2 be present in the strategically modified HBc protein molecule. It is further preferred when any residue is absent or deleted from position 1 to 10 that those residues be deleted in
15 sequence and that the remaining residues be present in sequence. Thus, if a five residue deletion were contemplated, the deleted residues would be numbered 1-5, leaving residues 6 through the desired HBc carboxy terminus present, plus the insert.

20 It is similarly preferred that residues numbered about position 140 through 149 of SEQ ID NO:2 be present in a strategically modified HBc protein molecule. As noted elsewhere herein, the region of HBc numbered 150 through the carboxy
25 terminus contains a plurality of arginine residues. Those residues bind nucleic acids on purification of HBc particles after expression, and the sequence containing those residues is preferably omitted from a strategically modified HBc protein molecule. As
30 was the case with the residues of positions 1 through 10, it is preferred that residues between about

-25-

position 140 and 149 be present and correspondingly absent in a sequential manner. Thus, where the carboxy terminal residue corresponds to the residue of position 146, the residues of positions 141-145 are also present and those of 147-149 are absent. Most preferably, a contemplated HBc sequence is that shown in SEQ ID NO:2 from position 1 through position 149, plus the sequence of the insert.

The insert can be placed within the HBc sequence in the region of positions numbered about 50 through about 100, as already noted. Preferably, the insert is present in the region corresponding to amino acid residues numbered about 70 to about 90. More preferably, the insert is present in the region corresponding to amino acid residues numbered 78 to 82. Most preferably, the insert is located in the region corresponding to residues numbered 78 through 80.

A strategically modified hepatitis B core protein of the invention has from 1 to about 40 amino acid residues inserted. Preferably, the insert is 1 to 10 amino acid residues in length. The insert contains a chemically-reactive amino acid residue. The insertion of more than one chemically-reactive amino acid residue is also contemplated.

It is contemplated that restriction endonuclease sites be provided in the gene construct for the strategically modified hepatitis B protein near the desired insert region. The nucleotides of the restriction endonuclease site will be translated into amino acids upon expression, and that effect has

-26-

some bearing on the choice of endonuclease. Several restriction endonucleases are commercially available (e.g. from Promega Corp., Madison, Wisconsin) and their recognition site sequences and cleavage sites well known in the art. Example 1 describes such a construct for a strategically modified hepatitis B core protein.

In one preferred embodiment, the insert is a single residue that is added as the chemically-reactive residue. In other preferred embodiments, the use of restriction enzymes and their recognition sequences causes about three to about five residues to be inserted, including the desired chemically reactive residue.

An insert containing a chemically-reactive amino acid residue is inserted into the native hepatitis B core protein at a position corresponding to an amino acid residue position from about 50 to about 100. The preferred region of insertion into the hepatitis B core protein is in the immunodominant loop region (about amino acid residue 70 to about 90), more preferably in the loop region that corresponds to the native hepatitis B core protein position from about amino acid 78 to about 82. Most preferably, the insert is placed at residues numbered 78 to 80 of SEQ ID NO:2.

As used herein when it is said that the insert is "at a position" it is meant that the amino terminus of the insert is peptide bonded to the carboxy terminus of the corresponding amino acid residue of the hepatitis B core protein sequence

-27-

having that amino acid residue number. In other words, the insert immediately follows the residue at that stated position.

Insertion can be effected by generally utilized methods in the art. Genetic manipulation, by a PCR-based method is illustrated in Examples 1 and 5. In addition, oligonucleotide-mediated site directed mutagenesis as discussed in J.Sambrook et al. Molecular Cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, 15.51-ff. (1989) can be used to add one codon by hybridizing a desired DNA sequence with a template that adds a codon for single residue, followed by filling in the remaining nucleic acid sequence. Dawson et al., Science 266:776-779 (1994), describe a method of linking polypeptide chains at their peptide backbone, so that a fusion could be built up of peptide fragments.

The chemically-reactive amino acid residue can be at any position within the insert. Preferably, the chemically-reactive amino acid residue is in a position that corresponds to amino acid residue numbered 70 to 90 of the native (wild type) core, and most preferably at residue position 78 to 82. For example, when a 10 amino acid residue long insert is inserted at a position corresponding to native core protein residue 73, then the chemically-reactive amino acid residue is preferably at position 5 to 9 of the insert. When a 30 amino acid residue long insert is inserted a position corresponding to native core protein residue 58, then

-28-

the chemically-reactive amino acid residue is preferably at position 22 to 24 of the insert.

An introduced chemically-reactive amino acid residue has a chemically-reactive side chain that provides a functional group for derivitizing the strategically modified HBc (i.e. conjugating a hapten to the modified HBc). Useful side chain functional groups include epsilon-amino groups, beta-or gamma-carboxyl groups, thiol (-SH) groups and aromatic rings (e.g. tyrosine and histidine). The chemically-reactive amino acid residue is typically a lysine, cysteine, or histidine residue or a carboxyl-containing residue such as aspartic acid or glutamic acid. Lysine is a particularly preferred chemically-reactive amino acid residue.

It is noted that the amino acid residue sequence of the hepatitis B core protein encoded by and shown in SEQ ID NOs:1 and 2, respectively, has two consecutive endogenous carboxyl-containing residues, existing glutamic (glu, E) and aspartic (asp, D) acids, at positions 77 and 78. However, the present invention contemplates the introduction of at least one *additional*, exogenous chemically-reactive amino acid residue. European Patent No. 385610 reports that unsatisfactory results were achieved in attempts to chemically couple polypeptide haptens to HBc particles. It is noted that those coupling attempts were directed toward amino groups and not carboxyl groups of the amino acid side chains.

In addition of the use of an individual chemically-reactive amino acid residue in the insert

-29-

such as aspartic acid or lysine, substantially any sequence of the desired length that contains a chemically-reactive amino acid residue can be used. Exemplary inserts of greater than a single residue include the B cell HRV-2 VP2 epitope discussed in B.E. Clarke et al. *Vaccines* 91:313-318 (1991), the HBsAg Pre-S(1)27-53 sequence discussed in F. Schödel et al. *J. Virol.* 66(1):106-114 (1992) and the HBsAg Pre-S(2)133-143 sequence discussed in F. Schödel et al. *Vaccines* 90:193-198 (1990). An appropriate T cell epitope discussed hereinafter as a hapten can also be used as an insert. Exemplary sequences include those of SEQ ID NOs: 28, 32, 33, 34, 47, 48, 49, 50 and 55.

15

ii. Additional Modification of the Core Protein

It is also contemplated that a hepatitis B core protein strategically modified as described above has other modifications. The contemplated modifications of the strategically modified core include the nature of the insert containing the chemically-reactive amino acid residue, truncation of the amino terminus, truncation of the carboxy terminus, fusion at the carboxy terminus, pendent linking to the carboxy-terminal region.

The insert containing the chemically-reactive amino acid residue to which the hapten is conjugated can have a use in addition to providing the chemically-reactive amino acid residue. It is contemplated that an insert containing a chemically-reactive amino acid residue is a T cell stimulating

-30-

amino acid sequence. Such a T cell stimulating amino acid sequence is preferably a T cell epitope from the same source as the B cell epitope that will be the conjugated antigen, e.g. both from *Mycobacterium*
5 *tuberculosis*. Exemplary epitopes are discussed hereinafter.

An insert containing a chemically-reactive amino acid residue can also be chosen in order to confer additional desirable properties, such as
10 stability-enhancing or solubility-enhancing properties.

A strategically modified hepatitis B core protein can be chemically modified by methods well known in the art. Numerous such techniques are
15 disclosed in Roger L. Lundblad, Techniques in Protein Modification, CRC Press (Ann Arbor, Michigan: 1994). Such chemical modifications are made to enhance or diminish properties, for example, a lysine amino group can be blocked to change the isoelectric point
20 of the protein, causing it to separate differently on a chromatographic ion exchange resin.

It is also contemplated that the carboxy terminus of the core protein sequence be truncated, preferably down to about amino acid residue position
25 140. The arginine-rich sequence present beginning at residue 150 of SEQ ID NO:2 binds to nucleic acids and can hinder the purification and handling of the expressed core protein. In SEQ ID NO:2, the arginine-rich stretches begin at position 150. A
30 preferred strategically modified HBc protein has a carboxy terminal valine (V) residue of residue 149.

-31-

iii. Making Strategically-Modified Core Protein

The strategic modification of the hepatitis B core protein is typically made by known processes in the art on the DNA level, for example by inserting the codons corresponding to the amino acids to be inserted. The engineered gene is then expressed in a convenient system known in the art, for example in a viral culture in infected immortalized cells.

Methods for producing HBcAg proteins in general and the pre-core, core and HBeAg proteins in particular, are well known in the art. The same methods readily adapted to the isolation of the modified core protein particles of the invention. In addition, HBcAg and HBeAg can be produced by a variety of well known recombinant DNA techniques. See, for example, U.S. Pat. No. 4,356,270 to Itakura and 4,563,423 to Murray et al., respectively. Those recombinant DNA techniques can be easily adapted to produce modified core particles of the invention. The modified core proteins can be conjugated with hapten before or after particle formation, preferably after core particle formation and purification.

C. Modified Hepatitis B Core Protein Conjugate

Any hapten against which antibody production is desired can be linked to a strategically modified hepatitis B core protein to form an immunogenic strategically modified hepatitis B core protein conjugate of this invention. The hapten of interest typically is a B cell determinant.

-32-

The hapten can be a polypeptide, a carbohydrate (saccharide), or a non-polypeptide, non-carbohydrate chemical such as 2,4-dinitrobenzene.

5 Methods for operatively linking individual haptens to a protein or polypeptide through an amino acid residue side chain of the protein or polypeptide to form a pendently-linked immunogenic conjugate, e.g., a branched-chain polypeptide polymer, are well known in the art. Those methods include linking
10 through one or more types of functional groups on various side chains and result in the carrier protein polypeptide backbone being pendently linked-- covalently linked (coupled) to the hapten but separated by at least one side chain.

15 Methods for linking carrier proteins to haptens using each of the above functional groups are described in Erlanger, *Method of Enzymology*, 70:85 (1980), Aurameas, et al., *Scand. J. Immunol.*, Vol. 8, Suppl. 7, 7-23 (1978) and U.S. Pat. No. 4,493,795 to
20 Nestor et al. In addition, a site-directed coupling reaction, as described in Rodwell et al., *Biotech.*, 3, 889-894 (1985) can be carried out so that the biological activity of the polypeptides is not substantially diminished.

25 Furthermore, as is well known in the art, both the HBcAg protein and a polypeptide hapten can be used in their native form or their functional group content can be modified by succinylation of lysine residues or reaction with cysteine-
30 thiolactone. A sulfhydryl group can also be incorporated into either carrier protein or conjugate

-33-

by reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(3-dithiopyridyl)propionate.

The HBc protein or hapten can also be
5 modified to incorporate a spacer arm, such as hexamethylene diamine or other bifunctional molecules of similar size, to facilitate the pendent linking.

Polypeptide hapten. Methods for covalent bonding of a polypeptide hapten are extremely varied
10 and are well known by workers skilled in the immunological arts. For example, following U.S. Patent No. 4,818,527, m-maleimidobenzoyl-N-hydroxysuccinimide ester (ICN Biochemicals, Inc.) or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-
15 carboxylate (SMCC, Pierce) is reacted with a strategically modified hepatitis B core protein to form an activated carrier. That activated carrier is then reacted with a polypeptide that either contains a terminal cysteine or to which an additional amino-
20 or carboxy-terminal cysteine residue has been added to form a covalently bonded strategically modified hepatitis B core protein conjugate. As an alternative example, the amino group of a polypeptide hapten can be first reacted with N-succinimidyl 3-(2-pyridylthio)propionate (SPDP, Pharmacia), and that
25 thiol-containing polypeptide can be reacted with the activated carrier after reduction. Of course, the sulfur-containing moiety and double bond-containing Michael acceptor can be reversed. These reactions
30 are described in the supplier's literature, and also in Kitagawa, et al., *J. Biochem.*, 79:233 (1976) and

in Lachmann et al., in 1986 Synthetic Peptides as Antigens, (Ciba Foundation Symposium 119), pp. 25-40 (Wiley, Chichester: 1986).

U.S. Patent No. 4,767,842 teaches several
5 modes of covalent attachment between a carrier and polypeptide that are useful here. In one method, tolylene diisocyanate is reacted with the carrier in a dioxane-buffer solvent at zero degrees C to form an activated carrier. A polypeptide hapten is
10 thereafter admixed and reacted with the activated carrier to form the covalently bonded strategically modified hepatitis B core protein conjugate.

Particularly useful are a large number of heterobifunctional agents that form a disulfide link
15 at one functional group end and a peptide link at the other, including N-succidimidyl-3-(2-pyridyldithio) propionate (SPDP). This reagent creates a disulfide linkage between itself and a thiol in either the strategically modified hepatitis B core protein or
20 the hapten, for example a cysteine residue in a polypeptide hapten, and an amide linkage on the coupling partner, for example the amino on a lysine or other free amino group in the carrier protein. A variety of such disulfide/amide forming agents are
25 known. See for example *Immun. Rev.* (1982) 62:185. Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thioether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic
30 acid, 2-bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl)cyclohexane-1-carboxylic acid and

-35-

the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt. The particularly preferred coupling agent for the method of this invention is succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) obtained from Pierce Company, Rockford, Ill. The foregoing list is not meant to be exhaustive, and modifications of the named compounds can clearly be used, e.g., the sulpho-SMCC depicted in the figure.

A polypeptide hapten can be obtained in a number of ways well known in the art. Usual peptide synthesis techniques can be readily utilized. For example, recombinant and PCR-based techniques to produce longer peptides are useful. Because the desired sequences are usually relatively short, solid phase chemical synthesis is useful.

As discussed below, DNA sequences that encode a variety of polypeptide haptens are known in the art. The coding sequence for peptides of the length contemplated herein can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., *J. Am. Chem. Soc.* 103:3185 (1981). Of course, by chemically synthesizing the coding sequence, any desired modification can be made simply by substituting the appropriate bases for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors now commonly available in the art,

-36-

and the regulating vectors used to transform suitable hosts to produce the desired protein.

A number of such vectors and suitable host systems are now available. For example promoter
5 sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. Typical of such vector plasmids are, for example, pUC8, and pUC13 available from J. Messing,
10 at the University of Minnesota (see, e.g., Messing et al., *Nucleic Acids Res.* 9:309 (1981)) or pBR322, available from New England Biolabs. Suitable promoters include, for example, the beta-lactamase (penicillinase) and lactose (lac) promoter systems
15 (Chang. et al., *Nature* 198:1056 (1977) and the tryptophan (trp) promoter system (Goeddel et al., *Nucleic Acids Res.* 8:4057 (1980)). The resulting expression vectors are transformed into suitable bacterial hosts using the calcium chloride method
20 described by Cohen, et al., *Proc. Natl. Acad. Sci. U.S.A.* 69:2110 (1972). Successful transformants may produce the desired polypeptide fragments at higher levels than those found in strains normally producing the intact pili. Of course, yeast or mammalian cell
25 hosts can also be used, employing suitable vectors and control sequences.

-37-

Table 2
Polypeptide haptens

Organism	Antigen	T or B cell epitope	Sequence	SEQ ID NO
<i>Streptococcus pneumoniae</i>	PspA	B	KLEELSDKIDELDAE	25
<i>Streptococcus pneumoniae</i>	PspA	B	SQKKYDEDQKKTEEKALEKA ASEEMDKAVAAVQQA	26
<i>Cryptosporidium parvum</i>	P23	B	QDKPADAPAAEAPAAEPAAQQ DKPADA	27
HIV	P24	T	GPKEPFRDYVDRFYKC	28
HIV	GP120	B	RKRIHIGPGRAPHYITKN	29
Foot and Mouth Disease Virus	VP1	B	YNGECRYNRNAVPNLRGDLQV LAQKVARTLP	30
<i>Corynebacterium diphtheriae</i>	toxin	T	FQVVHNSYNRPAYSPGC	31
<i>Borrelia burgdorferi</i>	OspA	T	VEIKEGTVTCLKREIDKNGKVT VSLC	32
<i>Borrelia burgdorferi</i>	OspA	T	TLKSNISKSGEVSVELNDC	33
Influenza A8/PR8	HA	T	SSVSSFERFEC	34
Influenza A8/PR8	HA	B	YRNLLWLTEK	35
<i>Yersinia pestis</i>	V Ag	B	DILKVIIVDSMNHGDAKSLR EELAEELTAEKLYSVIQAEIN KHLSSSGTINIHDKSINLMDK NLYGYTDEEIFKASAEYKILE KMPQTTIQVDGSEKKIVSIKD FLGSENKRTGALGNLKNSSYSY NKDNNELSHFATTCS	36
<i>Haemophilus influenzae</i>	pBOMP	B	CSSSNDAAGNGAAQFGGY	37
<i>Haemophilus influenzae</i>	pBOMP	B	NKLGTVSYGEE	38
<i>Haemophilus influenzae</i>	pBOMP	B	NDEAAYSKNRRVAVLAY	39
<i>Moraxella catarrhalis</i>	copB	B	LDIEKDKKKRTDEQLQAELEDD KYAGKGY	40
<i>Moraxella catarrhalis</i>	copB	B	LDIEKNKKKRTAELEQLAELEDD KYAGKGY	41
<i>Moraxella catarrhalis</i>	copB	B	IDIEKKGKIRTEAELELELNK DYPGQGY	42

Porphyromonas gingivalis	HA	B	GVSPKVCKDVTVEGSNEFAPV QNLT	43
Porphyromonas gingivalis	HA	B	RIQSTWRQKTVDLPAGTKYV	44
Trypanosoma cruzi		T	SHNFTLVASVIIIEEAPSGNTC	45
Trypanosoma cruzi		B	KAAIAPAKAAAAPAKAATAPA	46
Plasmodium falciparum	MSP1	T	SVQIPKVPYPNGIVYC	47
Plasmodium falciparum	MSP1	T	DFNHYYTLKTGLEADC	48
Streptococcus sobrinus	AgI/II	B & T	KPRPIYEAKLAQNQKC	49
Streptococcus sobrinus	AgI/II	B & T	AKADYEAKLAQYEKDLG	50
lymphocytic Choriomeningitis virus	NP	T	RPQASGVYMGNLTAQC	51
Shigella flexneri	Invasin	B	KDRTLIEQK	52
respiratory syncytial virus	G	B	CSICSNNPTCWAICK	53
Plasmodium vivax	CS	B	GDRADGQPAGDRADGQPAG	54
Clostridium tetani	tox	T	QYIKANSKFIGITELC	55
Entamoeba histolytica	lectin	B	VECASTVCQNDNSCPIIADVE KCNQ	56
Schistosoma japonicum	para	B	DLQSEISLSLENGELIRRAKS AESLASELQRRVD	57
Schistosoma mansoni	para	B	DLQSEISLSLENSELIRRAKA AESLASDLQRRVD	58
Plasmodium vivax		B	DRAAGQPAGDRADGQPAG	83
Influenza virus	Infl	B	CNNPHRIL	84
Influenza virus	Infl	T	CPKYVKQNTLKLATGMRNVPE KQTR	85
Influenza virus	Infl	B	SIMRSDAPIGTCSSECITPNG SIPNDKPFQNVNKITY	14
Influenza virus	Infl	B	RGIFGAIAGFIENGWEGMIDG WYGFRHQN	15
Influenza virus	Infl	B	EKQTRGIFGA	16
Mycobacterium tuberculosis		T	AVLEDPYILLVSSKV	86
Mycobacterium tuberculosis		T	LLVSSKVSTVKDLLP	87

Mycobacterium tuberculosis		T	LLPLLEKVVIGAGKPL	88
Mycobacterium tuberculosis		T	AILTGGQVISEEVGL	89
Mycobacterium tuberculosis		T	IAFNSGLEPGVVAEK	90
Mycobacterium tuberculosis		T	ARRGLERGLNALADAVKV	91
Mycobacterium tuberculosis		T	EKIGAELVKEVAKK	92
Mycobacterium tuberculosis		T	GLKRGIEKAVEKVTETL	93
Mycobacterium tuberculosis		T	IEDAVRNAKAAVEEG	94
Feline leukemia virus	FeLV	B	CDIIGNTWNPSDQEPFPGYG	95
Feline leukemia virus	FeLV	B	CIGTVPKTHQALCNETQQGHT	96
Feline leukemia virus	FeLV	B	GNYSNQTNPSPSC	97
Feline leukemia virus	FeLV	B	TDIQALEESISALEKSLTSLSE	98
Feline leukemia virus	FeLV		AKLRERLQKQQLF	99
Feline leukemia virus	FeLV		DSQQGWFEWGNKSPWFTTLISS	100
Feline leukemia virus	FeLV		QVMTITPPQAMGNLVLVP	101
Feline leukemia virus	FeLV		DQKPPSRQSQIESRVTP	102
Feline leukemia virus	FeLV		RRGLDILFLQEGGLC	103
Feline leukemia virus	FeLV		QEGGLCAALEECQIGGLCAALKEEC	104
Plasmodium falciparum		B	NANPNANPNANP	105
Circumsporozoite			QAQGDGANAGQP	113

Chemical Hapten. Related chemistry is used to couple chemical compounds to carrier proteins.

5 Typically, an appropriate functional group for coupling is designed into the chemical compound.

Antibodies to the compound 6-O-phosphocholine hydroxyhexanoate protect against *Streptococcus pneumoniae*. Randy T. Fischer et al. *J. Immunol.*, 154:3373-3382 (1995).

Table 3
Chemical Haptens

Chemical Hapten	Citation
piperidine N-oxide	U.S. Patent No. 5,304,252
phospholactone or lactamide	U.S. Patent No. 5,248,611
metal ion complexes	U.S. Patent No. 5,236,825
[2.2.1] or [7.2.2] bicyclic ring compounds	U.S. Patent No. 5,208,152
ionically charged hydroxyl-containing compounds	U.S. Patent No. 5,187,086
phosphonate analogs of carboxylate esters	U.S. Patent No. 5,126,258
cocaine analogs	Carrera et al., <i>Nature</i> 378:725 (1995)

5 *Carbohydrate Hapten.* There are many methods known in the art to couple carrier proteins to polysaccharides. Aldehyde groups can be generated on either the reducing end [Anderson, *Infect. Immun.*, 39:233-238 (1983); Jennings, et al., *J. Immunol.*, 127:1011-1018 (1981); Poren et al., *Mol. Immunol.*, 22:907-919 (1985)] or the terminal end [Anderson et al., *J. Immunol.*, 137:1181-1186 (1986); Beuvery et al., *Dev. Bio. Scand.*, 65:197-204 (1986)] of an oligosaccharide or relatively small polysaccharide,

10

-41-

which can be linked to the carrier protein via reductive amination.

Large polysaccharides can be conjugated by either terminal activation [Anderson et al., *J.*

5 *Immunol.*, **137**:1181-1186 (1986)] or by random activation of several functional groups along the polysaccharide chain [Chu et al., *Infect. Immun.*, **40**:245-256 (1983); Gordon, U.S. Patent 4,619,828 (1986); Marburg, U.S. Patent 4,882,317 (1989)].

10 Random activation of several functional groups along the polysaccharide chain can lead to a conjugate that is highly cross-linked due to random linkages along the polysaccharide chain. The optimal ratio of polysaccharide to carrier protein depend on the

15 particular polysaccharide, the carrier protein, and the conjugate used.

See Dick et al., in Contributions to Microbiology and Immunology, Vol. 10, Cruse et al., eds., (S. Karger: 1989), pp. 48-114; Jennings et

20 al., in Neoglycoconjugates: Preparation and Applications, Lee et al., eds., (Academic Press: 1994), pp. 325-371; Aplin et al., *CRC Crit. Rev. Biochem.*, **10**:259-306 (1981); and Stowell et al., *Adv. Carbohydr. Chem. Biochem.*, **37**:225-281 (1980) for

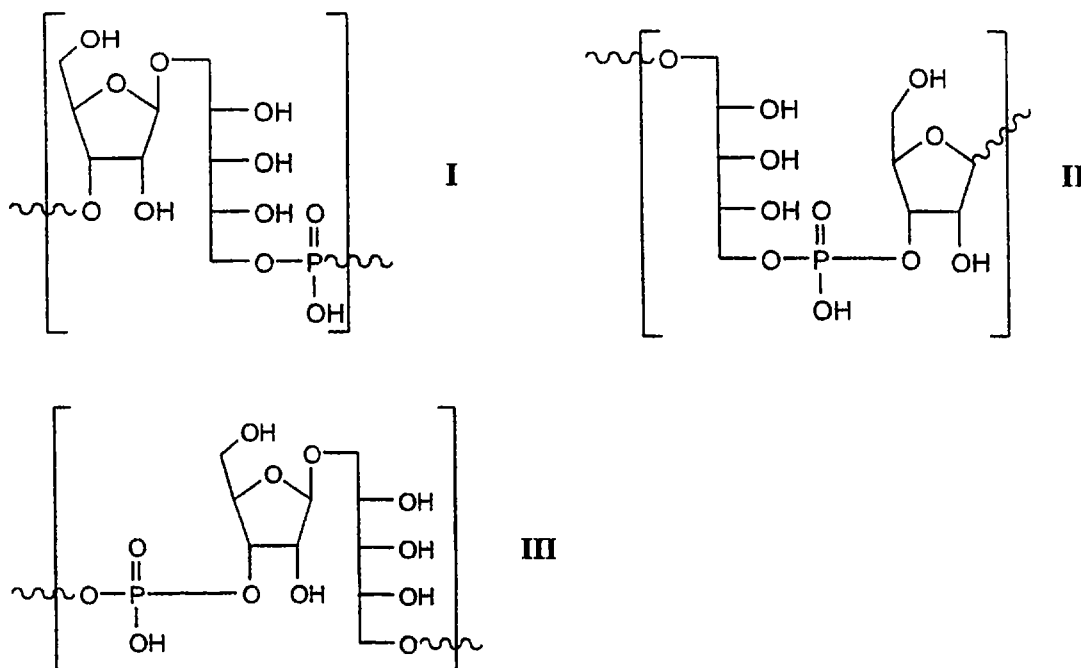
25 detailed reviews of methods of conjugation of saccharide to carrier proteins.

The carbohydrate itself can be synthesized by methods known in the art, for example by enzymatic glycoprotein synthesis as described by Witte et al.,

30 *J. Am. Chem. Soc.*, **119**:2114-2118 (1997).

Several oligosaccharides, synthetic and semi-synthetic, and natural, are discussed in the following paragraphs as examples of oligosaccharides that are contemplated haptens to be used in making a strategically modified core protein conjugate of the present invention.

An oligosaccharide hapten suitable for preparing vaccines for the treatment of *Haemophilus Influenza* type b (Hib) is made up of from 2 to 20 repeats of D-ribose-D-ribitol-phosphate (I, below), D-ribitol-phosphate-D-ribose (II, below), or phosphate-D-ribose-D-ribitol (III, below). Eduard C. Beuvery et al., EP-0 276 516-B1.



U.S. Patent 4,220,717 also discloses a polyribosyl ribitol phosphate (PRP) hapten for *Haemophilus influenzae* type b.

Ellena M. Peterson et al., *Infection and Immunity*, 66(8):3848-3855 (1998), disclose a trisaccharide hapten, α Kdo(2 8) α Kdo(2 4) α Kdo, that

provides protection from *Chlamydia pneumoniae*.
Chlamydia pneumoniae is a cause of human respiratory
infections ranging from pharyngitis to fatal
pneumonia. Kdo is 3-deoxy-D-manno-oct-2-ulosonic
5 acid.

Bengt Andersson et al., EP-0 126 043-A1,
disclose saccharides that can be used in the
treatment, prophylaxis or diagnosis of bacterial
infections caused by *Streptococci pneumoniae*. One
10 class of useful saccharides are derived from the
disaccharide GlcNAc β 1 3Gal. Andersson et al. also
found neolactotetraosylceramide to be useful, which
is Gal β 1 4GlcNAc β 1 3Gal β 1 4Glc-Cer.

European Patent No. 0 157 899-B1, the
15 disclosures of which are incorporated herein by
reference, discloses the isolation of pneumococcal
polysaccharides that are useful in the present
invention. The following table lists the
pneumococcal culture types that produce capsular
20 polysaccharides useful as haptens in the present
invention.

Table 4
Polysaccharide Hapten Sources

Danish Type Nomenclature	U.S. Nomenclature	1978 ATCC Catalogue Number
1	1	6301
2	2	6302
3	3	6303
4	4	6304
5	5	

-44-

6A	6	6306
6B	26	6326
7F	51	10351
8	8	6308
9N	9	6309
9V	68	
10A	34	
11A	43	
12F	12	6312
14	14	6314
15B	54	
17F	17	
18C	56	10356
19A	57	
19F	19	6319
20	20	6320
22F	22	
23F	23	6323
25	25	6325
33F	70	

Moraxella (*Branhamella*) *catarrhalis* is a known cause of otitis media and sinusitis in children and lower respiratory tract infections in adults.

5 The lipid A portion of the lipooligosaccharide surface antigen (LOS) of the bacterium is cleaved at the 3-deoxy-D-manno-octulosonic acid-glucosamine linkage. The cleavage product is treated with mild-alkali to remove ester-linked fatty acids while

10 preserving amide-linked fatty acids to yield

-45-

detoxified lipopolysaccharide (dLOS) from *M. catarrhalis*. The dLOS is not immunogenic until it is attached to a protein carrier. Xin-Xing Gu, et al. *Infection and Immunity* 66(5):1891-1897 (1998).

5 Group B streptococci (GBS) is a cause of sepsis, meningitis, and related neurologic disorders in humans. The Capsular polysaccharide-specific antibodies are known to protect human infants from infection. Jennings et al., U.S. 5,795,580. The
10 repeating unit of the GBS capsular polysaccharide type II is: 4)- β -D-GlcpNAc-(1 3)-[β -D-Galp(1 6)]- β -D-Galp(1 4)- β -D-Glcp-(1 3)- β -D-Glcp-(1 2)-[α -D-NeupNAc(2 3)]- β -D-Galp-(1 , where the bracketed portion is a branch connected to the immediately
15 following unbracketed subunit. The repeating unit of GBS capsular polysaccharide type V is: 4)-[α -D-NeupNAc-(2 3)- β -D-Galp-(1 4)- β -D-GlcpNAc-(1 6)]- α -D-Glcp-(1 4)-[β -D-Glcp-(1 3)]- β -D-Galp-(1 4)- β -D-Glcp-(1 .

20 European patent application No. EU-0 641 568-A1, Dr. Helmut Brade, discloses the method of obtaining ladder-like banding pattern antigen from *Chlamydia trachomatis*, *pneumoniae* and *psittaci*.

25 D. Pathogen-Related Conjugate to the Modified HBc

 In one embodiment of the invention, the hapten that is conjugated to strategically modified HBc protein is a B cell determinant of a pathogen. B cell determinants of numerous pathogens are known in
30 the art, and several were illustrated in the

-46-

preceding discussions of polypeptide and carbohydrate haptens.

In preferred embodiments, the hapten is a pathogen-related hapten. The use of a portion of a pathogen's protein sequence or carbohydrate sequence as a hapten has distinct advantages over the exposure to an actual pathogen, and even over a passivated or "killed" version of the pathogen.

Exemplary pathogen-related haptens of particular importance are derived from bacteria such as *B. pertussis*, *S. typosa*, *S. paratyphoid* A and B, *C. diptheriae*, *C. tetani*, *C. botulinum*, *C. perfringens*, *B. anthracis*, *P. pestis*, *P. multocida*, *V. cholerae*, *N. meningitides*, *N. gonorrhea*, *H. influenzae*, *T. palladium*, and the like.

Other exemplary sources of pathogen-related haptens of particular importance are viruses such as poliovirus, adenovirus, parainfluenza virus, measles, mumps, respiratory syncytical virus, influenza virus, equine encephalomyelitis virus, hog cholera virus, Newcastle virus, fowl pox virus, rabies virus, feline and canine distemper viruses, foot and mouth disease virus (FMDV), human and simian immunodeficiency viruses, and the like. Other important sources of pathogen-related haptens include rickettsiae, epidemic and endemic typhus, the spotted fever groups, and the like.

Pathogen-related polypeptide haptens are well-known in art and are discussed in numerous disclosures such as U.S. Pat. Nos. 3,149,036, 3,983,228, and 4,069,313; in Essential Immunology, 3rd

-47-

Ed., by Roit, published by Blackwell Scientific Publications; in Fundamentals of Clinical Immunology, by Alexander and Good, published by W.B. Saunders; and in Immunology, by Bellanti, published by W.B. Saunders.

Particularly preferred pathogen-related haptens are those described in U.S. Pat. Nos. 4,625,015, 4,544,500, 4,545,931, 4,663,436, 4,631,191, 4,629,783 and in Patent Cooperation Treaty International Publication No. WO87/02775 and No. WO87/02892, all of whose disclosures are incorporated herein by reference.

Antibodies that immunoreact with the hepatitis B virus can be obtained by using modified HBc conjugated with a polypeptide hapten that corresponds to the sequence of a determinant portion of HBsAg; in particular, residues 110-137 of the "S" (surface) region disclosed in Gerin et al., *Proc. Natl. Acad. Sci. USA*, 80:2365 (1983).

Another conjugate corresponds to amino acids 93-103 of capsid protein VPI of poliovirus type 1 (PV1, Mahoney strain), analogous to the work by Delpeyroux et al., *Science*, 233:472-475 (1986). Such a modified HBc conjugate provides antibodies that immunoreact with polio. Other potential haptens from poliovirus type 1, Mahoney and Sabin strains are described in European Patent No. 385610.

In preferred embodiments, the hapten is a pathogen-related hapten that immunoreacts with; i.e., is immunologically bound by, antibodies induced by the pathogen. More preferably, the pathogen-related

-48-

hapten induces an antibody response that provides protection against infection by the pathogen.

Methods for determining the presence of antibodies to an immunogen in a body sample from an immunized animal are well known in the art. Methods for determining the presence of both cross-reactive and protective antibodies are well known in the art.

In another embodiment of the invention, the immune response to the B cell determinant is boosted by also providing a T cell determinant.

For example, U.S. Patent No. 4,882,145 describes T cell stimulating polypeptides derived from the HBV nucleocapsid protein. Other T cell determinants are known in the art and can be used as an operatively linked determinant in a contemplated modified HBc protein or particle.

In a particularly preferred embodiment of the invention, such a T cell determinant is derived from the same pathogen as the B cell determinant that is conjugated to the modified HBc. The T cell determinants of various pathogens are reported in the art.

Although it is preferred that the B and T cell determinants are derived from the same pathogen, it is not necessary that they be from the same protein of that pathogen. For example, the B cell determinant from the VP1 protein of the foot and mouth disease virus (FMDV) can be conjugated to a modified HBc particle, wherein the HBc protein is further modified by having a T cell determinant derived from the VP4 protein of FMDV.

The additional T cell determinant can be introduced into a modified HBc protein on the genetic level using well-known methods within or at a terminus of the HBc protein sequence (amino or carboxy termini), including as part of the DNA inserted to introduce the chemically-reactive amino acid residue, but preferably as a fusion protein at the carboxy terminus. Alternatively, the additional T cell determinant can be operatively linked to the conjugated B cell determinant or the strategically modified HBc protein.

Exemplary disclosures that describe techniques for genetically engineering a DNA sequence that can be used to produce a fusion protein of the present invention can be found in: U.S. Pat. Nos. 4,428,941 to Galibert et al., 4,237,224 to Cohen et al.: 4,273,875 to Manis; 4,431,739 to Riggs; 4,363,877 to Goodman et al., and Rodriguez & Tait, *Recombinant DNA Techniques: An Introduction*, The Benjamin-Cummings Publishing Co., Inc. Menlo Park, Calif. (1983), whose disclosures are incorporated by reference.

E. Inocula and Vaccines

In yet another embodiment of the invention, a modified HBc protein or particle conjugated to a hapten (a HBc conjugate) is used as the immunogen of an inoculum that induces production of antibodies that immunoreact with the hapten or as a vaccine to provide protection against the pathogen from which the hapten is derived.

-50-

A contemplated inoculum or vaccine comprises a HBcAg conjugate that is dissolved or dispersed in a pharmaceutically acceptable diluent composition that typically also contains water. When
5 administered in an immunogenic effective amount to an animal such as a mammal (e.g., a mouse, dog, goat, sheep, horse, bovine, monkey, ape, or human) or bird (e.g., a chicken, turkey, duck or goose), an inoculum induces antibodies that immunoreact with the
10 conjugated (pendently-linked) hapten. A vaccine is a type of inoculum in which the hapten is pathogen related and the induced antibodies not only immunoreact with the hapten, but also immunoreact with the pathogen or diseased cell, and neutralize
15 the pathogen or diseased cell with which they immunoreact.

The preparation of inocula and vaccines that contain proteinaceous materials as active ingredients is also well understood in the art.
20 Typically, such inocula or vaccines are prepared as parenterals, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.
25 The immunogenic active ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations
30 thereof. In addition, if desired, an inoculum or vaccine can contain minor amounts of auxiliary

-51-

substances such as wetting or emulsifying agents, pH buffering agents or adjuvants which enhance the immunogenic effectiveness of the composition.

Exemplary adjuvants include complete Freund's adjuvant (CFA) that is not used in humans, incomplete Freund's adjuvant (IFA) and alum, which are materials well known in the art, and are available commercially from several sources. The use of small molecule adjuvants is also contemplated herein.

Exemplary of one group of small molecule adjuvants are the so-called muramyl dipeptide analogues described in U.S. Patent No. 4,767,842. Another type of small molecule adjuvant described in U.S. Patent No. 4,787,482 that is also useful herein is a 4:1 by volume mixture of squalene or squalane and Aracel™ A (mannide monooleate).

Yet another type of small molecule adjuvant useful herein is a 7-substituted-8-oxo or 8-sulfo-guanosine derivative described in U.S. Patents No. 4,539,205, No. 4,643,992, No. 5,011,828 and No. 5,093,318, whose disclosures are incorporated by reference. Of these materials, 7-allyl-8-oxoguanosine (loxoribine) is particularly preferred. That molecule has been shown to be particularly effective in inducing an antigen-(immunogen-)specific response

Inocula and vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations that are suitable for other modes of

-52-

administration include suppositories and, in some cases, oral formulation or by nasal spray. For suppositories, traditional binders and carriers can include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range 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.

An inoculum or vaccine composition takes the form of a solution, suspension, tablet, pill, capsule, sustained release formulation or powder, and contains an immunogenic amount of strategically modified HBc protein conjugate or strategically modified HBc protein particle conjugate as active ingredient. In a typical composition, an immunogenic amount of strategically modified HBc protein conjugate or strategically modified HBc protein particle conjugate is about 50 μ g to about 2 mg of active ingredient per dose, and more preferably about 100 μ g to about 1 mg per dose.

The particles and protein conjugates can be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic,

-53-

oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The inocula or vaccines are administered in a manner compatible with the dosage formulation, and in such amount as are 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 judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in intervals (weeks or months) by a subsequent injection or other administration.

Another embodiment of the invention is a process for inducing antibodies in an animal host comprising the steps of inoculating said animal host with an inoculum. The inoculum used in the process comprises an immunogenic amount of a strategically modified hepatitis B core protein conjugate dissolved or dispersed in a pharmaceutically acceptable

-54-

diluent. The strategically modified hepatitis B core protein conjugate used in the process comprises a hapten pendently linked to a strategically modified hepatitis B core protein. Preferably the

5 strategically modified hepatitis B core protein is in particle form. The strategically modified hepatitis B core protein comprises an amino acid sequence corresponding to the hepatitis B core protein amino acid sequence of SEQ ID NO:2 including the amino acid

10 residues numbered about 10 to about 140 and additionally having an insert in the region corresponding to amino acid residues numbered about 50 to about 100, said insert (i) being 1 to about 40 amino acid residues in length, and (ii) containing a

15 chemically-reactive amino acid residue. The hapten is pendently linked to the strategically modified hepatitis B core protein through said chemically-reactive amino acid residue. Preferably, the hapten is pathogen-related. The animal is maintained for a

20 time sufficient for antibodies to be induced.

The invention is illustrated by the following non-limiting examples.

25 Example 1: Construction of a Modified Hepatitis B
Core Protein Expression Vector

Using site-directed mutagenesis, a lysine codon (TTT) was introduced between amino acids D78 and P79 of the HBc gene, along with EcoRI (GAATTC)

30 and SacI (GAGCTC) restriction endonuclease sites to facilitate the genetic insertion of other condons for

-55-

producing strategically modified hybrid HBc particles. The insert thus had an amino acid residue sequence GIQKEL, where the GIQ is an artifact of the EcoRI site and the EL is an artifact of the SacI site. The strategically modified hepatitis B core protein was therefore 155 amino acid residues long. The construction of the pKK322-HBc155-K81 expression plasmid is described below.

Oligonucleotide primers **P1F** (SEQ ID NO:17) and **P1R** (SEQ ID NO:18, on the complementary strand) were used to amplify the 5' end of the HBc gene (bases 1-234, amino acids 1-78), and simultaneously incorporate an NcoI restriction site (CCATGG) at the 5' end, and an EcoRI restriction site (GAATTC) at the 3' end of the amplified product. Oligonucleotide primers (SEQ ID NO:19) **P2F** and **P2R** (SEQ ID NO:20, on the complementary strand) were used to amplify the 3' end of the HBc gene (bases 232-450, amino acids 79-149), and simultaneously incorporate an EcoRI restriction site (GAATTC) at the 5' end, a SacI restriction site (GAGCTC) adjacent to it, an inserted lysine codon (AAA) between them, and a HindIII restriction site at (AAGCTT) the 3' end of the amplified product.

The two PCR products (encoding amino acids 1-78 and amino acids 79-149) were cleaved with EcoRI, ligated together at their common EcoRI overhangs, cleaved with NcoI and HindIII and cloned into the expression plasmid pKK332 (Pharmacia), using standard techniques. The resulting plasmid was called pKK332-HBc-K81. This plasmid can be used for the expression

-56-

of a strategically modified HBc protein bearing a lysine in the immunodominant loop. The expressed strategically modified HBc protein spontaneously formed particles. The strategically modified HBc of this Example thus had an insert corresponding to position 78 of the HBc of SEQ ID NO:2, a chemically reactive lysine residue at a position corresponding to position 82 of the HBc of SEQ ID NO:2, and was truncated at a position corresponding to position 149 of the HBc of SEQ ID NO:2.

-57-

Example 2: Modified Hepatitis B Core

Particle Purification

Strategically modified HBc particles of Example 1 were expressed in E. coli typically E. coli BLR or BL21 from Novagen (Madison, Wisconsin) or E. coli TB11 from Amersham (Arlington Heights, Illinois). The transfected E. coli ~~denoted~~ HBc155-K81, were expressed plasmid pKK332-HBc155-K81. The strategically modified HBc particles were purified via Sepharose CL-4B chromatography using established procedures. Because particles purify in a predictable manner, the monitoring of particle elution using simple spectroscopy (OD_{280}), in concert with SDS-PAGE analysis to assess purity of individual fractions prior to pooling, was sufficient to enable the routine purification of electrophoretically pure particles in high yield (5-120 mg/L cell culture). The spherical structure of the pure strategically modified hepatitis B core particles was clearly visible in an electron micrograph.

Example 3: Chemical Coupling of Synthetic Peptides and Modified Hepatitis B Core Particles

The strategically modified hepatitis B core particle product of the expression plasmid pKK332-HBc155-K81 from Example 1 was assayed for its chemical reactivity compared with similarly expressed and purified "wild type" truncated hepatitis B core particle HBc149, which is identical to HBc155-K81

except that it lacks the introduced lysine residue and flanking five amino acids.

Synthetic peptides (haptens) were chemically conjugated to HBC particles using succinimidyl 4-(N-maleimido-methyl) cyclohexane 1-carboxylate (SMCC), a water-soluble heterobifunctional cross-linking reagent. SMCC is reactive towards both sulfhydryl and primary amino groups, enabling the sequential conjugation of synthetic peptides to HBC particles whose primary amino groups have previously been modified with SMCC. Further, the 11.6 angstrom spacer arm afforded by SMCC helps to reduce steric hindrance between the hapten and the HBC carrier, thereby enabling higher coupling efficiencies.

Briefly, HBc155-K21 and HBc149 particles were separately reacted with a 3-fold excess of SMCC over total amino groups (native amino groups or native amino groups plus the one from the lysine residue of the insert) for 2 hours at room temperature in 50 mM sodium phosphate, pH 7.5, to form maleimide-activated HBC particles. Unreacted SMCC was removed by repeated dialysis against 50 mM sodium phosphate, pH 7.0. The SMCC derivatization of the HBC particles resulted in a minimal molecular weight increase which was not detectable by SDS-PAGE. However, the PAGE analysis did confirm the integrity of the HBC proteins prior to proceeding to the peptide conjugation step.

Synthetic peptides to be coupled to the HBC particles were designed such that they had N-terminal

-59-

cysteine residues to enable directional conjugation of peptide haptens to the primary amine on the side chain of the introduced lysine residue via the cysteine sulfhydryl of the hapten.

5 Table 2 shows the synthetic peptides derived from human cytochrome P450 enzymes that were chemically conjugated to HBc particles. The synthetic peptides were dissolved in 50 mM sodium phosphate, pH 7.0, to a concentration of 10 mg/ml.

10 The synthetic peptides were then added, dropwise, to a 5-fold excess over total amino groups in maleimide-activated strategically modified HBc155-K81 particles, and permitted to react at room temperature for 2 hours. Maleimide-activated HBc149 particles

15 were reacted with the two 2D6 peptides (206 and 206-C) as controls.

Table 5
Cytochrome P-450 Haptens

	Peptide Name	Sequence	SEO ID NO.:
5	1A1 (289-302)	CQEKQLDENANVQL	21
	1A2 (291-302)	CSKKGPRASGNLI	22
	2D6 (263-277)	CLTEHRMTWDPAQPPRDLT	23
	3A4 (253-273)	CVKRMKESRLEDTQKHRVDFLQ	24
	1A1-c	CMQLRS	106
10	1A2-c	CRFSIN	107
	2D6-c	CAVPR	108
	2E1-c	CIPRS	109
	2C-c	CFIPV	110
	3A3/4/7-c	CTVSGA	111
15	3A5-c	CTLSGE	112

Example 4: Analysis of Modified Core
Particles Conjugates

20 Strategically modified HBc particles
pendently linked to cytochrome P-450 determinant
haptens of Example 3 were analyzed by SDS-PAGE and
immunoblots to determine if synthetic peptides had
been successfully conjugated to HBc. The denaturing
25 conditions of the electrophoresis dissemble of
particles into their constituent subunits, HBc
monomers. Because HBc monomers have a molecular
weight of approximately 16,000 Da, it was simple to
resolve HBc155-K81 particles chemically conjugated to
30 either 1A1 (289-302), 1A2 (291-302), 2D6 (263-277) or
3A4 (253-273) peptides, as those peptides have a

-61-

relative molecular mass of approximately 2,000 Da and therefore cause a visible increase in the molecular mass of the HBc protein monomers. From the relative intensities of the conjugated and non-conjugated bands on SDS-PAGE, it was revealed that approximately 50 percent of the HBc155-K81 monomers were operatively linked to hapten, whereas only about 5 percent of the "wild type" HBc149 particles were linked to hapten. The marked increase in the observed success in pendently linking hapten to the strategically modified hepatitis B core protein supports the conclusion that the observed linking occurs via the inserted lysine as opposed to a lysine residue that is also present in the "wild type".

The shift in mobility of HBc particles conjugated to shorter C-terminal P450 derived peptides (5 and 6-mers) is not as pronounced in the SDS-PAGE as that of the longer inhibitory peptides, but shifts of approximately 700 Da were clearly evident in successfully coupled HBc155-K81 monomers. The strategically modified HBc 155-K81 protein exhibited markedly enhanced ability to pendently link to a hapten over the "wild type" HBc149 particles, which showed minimal conjugation.

In the model of core particles propounded by Conway et al. of icosahedral particles of either 180 or 240 associated core protein monomers [Nature, 386:91-94 (1997)], dimers of the relatively exposed immunodominant loop regions of the core monomers extend out from the assembled core particle into solution like spikes on a mace. The "spikes" are

-62-

closely arranged spatially on the HBc particles. The strategic location of the introduced lysine residue on the tip of the spike minimizes the propensity for steric constraints to reactions to link haptens to the assembled core particle. A maximum of 50 percent of the strategically modified HBc monomers were successfully conjugated to the synthetic peptides of Cyt P-450. That amount of pendent linkage corresponds to an average of one hapten attached per core particle spike. This proposed distribution of hapten linkage to the strategically modified HBc particle is supported by PAGE results under semi-denaturing conditions that disassemble the particle while maintaining the dimer association.

HBc-2D6 particles, prepared by peptide coupling, were examined using immunoblots to confirm the presentation of the 2D6 epitope. When probed with anti-HBc antisera, the chemically coupled particle yielded two different monomers representing particles with and without the 2D6 peptide. Only the upper band of which blotted with anti-2D6 antisera, thereby confirming the correlation between mobility shift and attachment of the 2D6 peptide.

Example 6: Strategic Lysine Insertions

To construct HBc particles with inserted lysine residues at every position in the immunodominant, surface-exposed loop region (amino acids 75-85), PCR was used to amplify the 5' and 3' fragments of the HBc gene and a single lysine codon was introduced via the oligonucleotide primers. The

8 AUG 2002

oligonucleotide primers and the resulting amino acid sequences are shown in SEQ ID NOs: 61-82. The "wild type" sequences are SEQ ID NOs:59-60.

In order to generate lysine inserts at positions 75 to 84 (HBc-K75 through HBc-K84), the pairs of PCR fragments were digested with the restriction endonuclease *MseI*, which recognizes the sequence, AATT. The modified gene was restored by ligating the oligonucleotide primer (containing the lysine) at the convenient *MseI* restriction site located at nucleotides 221-224. For HBc-K85 (SEQ ID NOs:85-86) it was necessary to generate two fragments that were ligated at a common *XhoI* restriction site (CTCGAG) that is not present in the wild type gene, but could be introduced at position 239-244 without altering any amino acids.

Table 6

Lysine insertion mutants of HBc
in the immunodominant loop

Name	Sequence	SEQ ID NO:
Wild Type HBc	TWVGVNLEDPASRDLVVSIV	60
K75	TWVGVNLEDPASRDLVVSIV	62
K76	TWVGVNKLEDPASRDLVVSIV	64
K77	TWVGVNLKEDPASRDLVVSIV	66
K78	TWVGVNLEKDPASRDLVVSIV	68
K79	TWVGVNLEDKPPASRDLVVSIV	70
K80	TWVGVNLEDPKASRDLVVSIV	72
K81	TWVGVNLEDPAKSRDLVVSIV	74
K82	TWVGVNLEDPAKSRDLVVSIV	76

-64-

K83	TWVGVNLEDPASR <u>K</u> DLVVSYV	78
K84	TWVGVNLEDPASRD <u>K</u> LVVSYV	80
K85	TWVGVNLEDPASRDL <u>K</u> VVSYV	82

To purify the strategically modified HBc proteins, cleared cell lysates from a 1L fermentation were precipitated with 45% ammonium sulfate and the resultant pellet subjected to gel filtration using Sepharose CL-4B chromatography (2.5cm x 100cm). Particulate HBc has a characteristic elution position when analyzed using this type of column, which was independent of the amino acid insertions made to the particle. The eleven strategically modified HBc particles generated for this study were analyzed using this procedure, and the elution profiles were measured spectrophotometrically at an absorbance of 280 nm.

Three of the constructs (HBc-K75, HBc-K77, and HBc-K79) were produced at levels of between 50 and 100 mg/L, which is comparable with typical yields for wild-type, unmodified HBc particles, e.g. HBc149 particles. Four of the constructs (HBc-K76, HBc-K78, HBc-K81, and HBc-K82) were produced at relatively low levels of between 1 and 20 mg/L. Finally, four of the particles (HBc-K80, HBc-K83, HBc-K84, and HBc-K85) were produced at levels deemed to be barely detectable (< 1 mg/L).

-65-

Table 7

Yields of purified lysine-containing
HBc particles from a 1L fermentation

Particle	Yield(mg/L)
HBc-150 (K75)	77
HBc-150 (K76)	5
HBc-150 (K77)	74
HBc-150 (K78)	10
HBc-150 (K79)	94
HBc-150 (K80)	0
HBc-150 (K81)	17
HBc-150 (K82)	1
HBc-150 (K83)	0
HBc-150 (K84)	0
HBc-150 (K85)	0

5

The foregoing description of the invention,
including the specific embodiments and examples, is
intended to be illustrative of the present invention
and is not to be taken as limiting. Numerous other
variations and modifications can be effected without
departing from the true spirit and scope of the
present invention.

10

8 AUG 2002

-66-

What is claimed:

1. A strategically modified hepatitis B
core protein conjugate comprising a strategically
5 modified hepadnavirus nucleocapsid protein pendently
linked to a hapten, wherein the strategically
modified hepatitis B core protein comprises an amino
acid sequence corresponding to the hepatitis B core
protein amino acid sequence of SEQ ID NO:2 including
10 the amino acid residues numbered about 10 to about
140 and additionally having an insert in the region
corresponding to amino acid residues numbered about
50 to about 100, said insert (i) being 1 to about 40
amino acid residues in length, and (ii) containing a
15 chemically-reactive amino acid residue, said hapten
being pendently linked to said chemically-reactive
amino acid residue.
2. The strategically modified hepatitis B
20 core protein conjugate according to claim 1 wherein
the insert is in the region corresponding to amino
acid residues numbered about 70 to about 90.
3. The strategically modified hepatitis B
25 core protein conjugate according to claim 1 wherein
the insert is in the region corresponding to amino
acid residues numbered 78 to 82.
4. The strategically modified hepatitis B
30 core protein conjugate according to claim 1 wherein

-67-

the hapten is a polypeptide hapten, chemical hapten or carbohydrate hapten.

5 5. The strategically modified hepatitis B core protein conjugate according to claim 1 wherein the hapten is a pathogen-related hapten.

10 6. The strategically modified hepatitis B core protein conjugate according to claim 5 wherein the strategically modified hepatitis B core protein further comprises a T cell stimulating amino acid residue sequence operatively linked to the carboxy terminus of said hepatitis B amino acid sequence.

15 7. The strategically modified hepatitis B core protein conjugate according to claim 6 wherein said T cell stimulating amino acid residue sequence is related to the same pathogen as the pendently linked pathogen-related hapten.

20 8. A strategically modified hepatitis B core protein particle comprising a strategically modified hepatitis B core protein subunit having an insert containing a chemically-reactive amino acid side chain, said insert not itself an antigenic determinant.

30 9. A strategically modified hepatitis B core protein particle conjugate comprising a plurality of strategically modified hepatitis B core protein subunits pendently linked to a hapten.

10. The strategically modified hepatitis B core protein particle conjugate according to claim 9 wherein about 0.1 to about 0.5 of the strategically modified hepatitis B core protein subunits are pendently linked to a hapten.

11. The strategically modified hepatitis B core protein particle conjugate according to claim 9 having a plurality of strategically modified hepatitis B core protein subunits that are pendently linked to different haptens.

12. The strategically modified hepatitis B core protein particle conjugate according to claim 9 wherein the strategically modified hepatitis B core protein subunit further comprises a T cell stimulating amino acid residue sequence peptide-bonded to the carboxy terminal residue of the amino acid sequence corresponding to said hepatitis B amino acid sequence.

13. The strategically modified hepatitis B core protein particle conjugate according to claim 12 wherein the T cell stimulating amino acid residue is from the same pathogen as the pendently linked hapten.

14. An immunogenic fusion protein conjugate comprising a polypeptide hapten pendently linked to a strategically modified hepatitis B core

-69-

protein that comprises three peptide-linked domains,
I, II and III from the N-terminus, wherein

(a) Domain I corresponds to residues about
10 through 50 of the amino acid sequence of hepatitis
5 B core protein of SEQ ID NO:2;

(b) Domain II is bonded to the carboxy
terminal residue of Domain I and corresponds to
residues 50 to 100 of said amino acid sequence of
hepatitis B core protein that further contains a
10 heterologous insert 1 to about 40 amino acid residues
in length that includes a chemically-reactive amino
acid residue; and

(c) Domain III is bonded to the carboxy
terminal residue of Domain II and corresponds to
15 residues 100 to about 140 of the amino acid sequence
of hepatitis B core protein.

15. An immunogenic fusion protein
conjugate according to claim 14 wherein the
20 strategically modified hepatitis B core protein
further comprises a fourth peptide-linked domain,
Domain IV, wherein

(d) Domain IV is bonded to the carboxy
terminal residue of Domain III and comprises a T cell
25 epitope.

16. A strategically-modified hepatitis B
core protein comprising an amino acid sequence
corresponding to about amino acid residue 10 to about
30 amino acid residue 140 of the hepatitis B amino acid
sequence of SEQ ID NO:2 and having an amino acid

-70-

sequence insert in the region corresponding to about
residue 50 to about residue 100 of the hepatitis B,
wherein the insert includes a chemically-reactive
amino acid residue and the insert itself is not an
5 antigenic determinant.

17. An inoculum comprising an immunogenic
amount of a strategically modified hepatitis B core
protein conjugate dissolved or dispersed in a
10 pharmaceutically acceptable diluent, said
strategically modified hepatitis B core protein
conjugate comprising a hapten pendently linked to a
strategically modified hepatitis B core protein,
wherein the strategically modified hepatitis B core
15 protein comprises an amino acid sequence
corresponding to the hepatitis B core protein amino
acid sequence of SEQ ID NO:2 including the amino acid
residues numbered about 10 to about 140 and
additionally having an insert in the region
20 corresponding to amino acid residues numbered about
50 to about 100, said insert (i) being 1 to about 40
amino acid residues in length, and (ii) containing a
chemically-reactive amino acid residue, said hapten
being pendently linked to said chemically-reactive
25 amino acid residue.

18. The inoculum according to claim 17
wherein said strategically modified hepatitis B core
protein conjugate is present in the form of
30 particles.

-71-

19. The inoculum according to claim 18 wherein said hapten is from a pathogen and wherein use of said inoculum in a mammalian host protects said mammalian host from said pathogen.

5

20. A process for inducing antibodies in an animal host comprising the steps of inoculating said animal host with an inoculum that comprises an immunogenic amount of a strategically modified hepatitis B core protein conjugate dissolved or
10 dispersed in a pharmaceutically acceptable diluent, said strategically modified hepatitis B core protein conjugate comprising a hapten pendently linked to a strategically modified hepatitis B core protein,
15 wherein the strategically modified hepatitis B core protein comprises an amino acid sequence corresponding to the hepatitis B core protein amino acid sequence of SEQ ID NO:2 including the amino acid residues numbered about 10 to about 140 and
20 additionally having an insert in the region corresponding to amino acid residues numbered about 50 to about 100, said insert (i) being 1 to about 40 amino acid residues in length, and (ii) containing a chemically-reactive amino acid residue, said hapten
25 being pendently linked to said strategically modified hepatitis B core protein through said chemically-reactive amino acid residue, and maintaining said animal for a time sufficient for antibodies to be induced.

30

8 AUG 2002

8 AUG 2002

21. The process according to claim 20 wherein said strategically modified hepatitis B core protein conjugate is present in the form of particles.

5

22. The process according to claim 21 wherein said hapten is from a pathogen and wherein the antibodies induced in said mammalian host protect said mammalian host from said pathogen.

10