METHOD FOR TREATING CHRONIC HBV INFECTION

FIELD OF THE INVENTION

The present invention relates to an immunotherapeutic for the treatment of chronic Hepatitis B virus ("HBV") infection and disease.

BACKGROUND OF THE INVENTION

Greater than 85% of childhood HBV infections lead to chronicity. One to five % of acute HBV infections in adults fail to resolve, also leading to chronic infection. Chronic infection can lead to chronic active hepatitis (20-50% of the cases), cirrhosis of the liver or, even, liver failure (10-20% of the cases), and, in some instances, liver cancer (1-3% of the cases). Liver injury results primarily from cellular immune responses to infected hepatocytes.

In the United States alone, there are approximately 1 million chronic carriers of HBV. Worldwide, there are over 300 million chronic carriers. Many of the chronic carriers are found along the Pacific Rim, i.e., Taiwan, Japan, Southeast Asia, and Africa.

The mechanisms responsible for chronic HBV infection are unclear. The presence of Hepatitis B e-antigen ("HBeAg" or "HBe", pre-core polypeptide) is associated with active viral replication and is indicative of increased infectiousness and severity. Although prognosis is better if the patient is anti-HBe positive, the resolution of both acute and chronic infection is associated with the development of antibodies against Hepatitis B surface antigen ("HBsAg" or "HBs"). HBsAg loss with the appearance of neutralizing anti-HBs antibodies is indicative of clearance of infection. Spontaneous seroconversion to anti-HBs, however, is extremely rare.

While approximately 10 % of the patients per year experience spontaneous seroconversion from HBeAg to anti-HBe, seroconversion from HBs to anti-HBs occurs in only about 1 % of the patients per year.

Although therapies for chronic HBV are available, most are limited both in scope and efficacy. Interferon therapy leads to anti-HBs seroconversion in only 3-5 % of the patients. Additionally, interferon therapy is very expensive, can have severe side effects, and requires daily injections sub-cutaneously.

Newer antiviral agents, such as lamivudine, can reduce viral loads, but lead to anti-HBs seroconversion in only a few patients. Further, they must be used long-term -- discontinuation leads to the reappearance of the virus, making the requirement for lifetime treatment a possibility -- and resistant mutants can emerge.

Immunotherapies are also available. Some immunotherapies are based upon the administration of antibody. Anti-HBs antibody ("HBIG"), when used alone, can have therapeutic activity, as evidenced in liver transplantation for HBV infection. Protein Design Labs is pursuing the use of a humanized anti-HBs antibody for Ig infusion therapy.

Other immunotherapies are based upon the administration of antigen. One antigen-based immunotherapy utilizes yeast-expressed HBsAg with an oil/water emulsion with RIBI® and QS-21 (SmithKline Beecham). It is currently in Phase 2 trial for chronic infection. Another utilizes PreS1 + PreS2 + S/alum (Medeva). Still another, currently in an 150 subject immunotherapy trial, uses PreS2+S/alum (Institut Pasteur). Another antigen-based immunotherapy utilizes core-derived peptide for the purpose of inducing a cytotoxic T lymphocyte response (Cytel).

The need for a potent immunotherapy that can ameliorate chronic HBV infection remains.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a method for treating chronic HBV infection comprising the administration of a composition comprising recombinant PreS2+S protein of HBsAg and a metabolizable oil adjuvant and,

optionally, an anti-viral agent or compound.

In a further aspect, the present invention relates to a kit for immunotherapy comprising PreS2 + S protein of HBsAg, a metabolizable oil adjuvant, and, optionally, an anti-viral agent or compound.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the HBV genome with restriction sites of interest and the adaptors for preparing the expression plasmid.

Figure 2 schematically depicts the cloning strategy.

Figures 3a-b, 4a-b, and 5 a-b depict the HBe/Anti-HBe(a) and serum ALT/HBV DNA (b) results for three patients, the second exhibiting a transient flare.

DETAILED DESCRIPTION

The practice of the present invention will employ, unless otherwise indicated, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *DNA Cloning: A Practical Approach*, Vols. I & II (D. Glover, ed.); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications); and *Fundamental Virology*, 2nd Edition, Vols. I & II (B.N. Fields and D.M. Knipe, eds.).

The present invention is directed to an antigen-based immunotherapy for treating chronic HBV infection. As used herein, "treating" includes the amelioration and/or elimination of chronic HBV infection, as well as the stimulation of an immune response thereto, preferably an anti-HBsAg or anti-HBe response. Human HBV is a member of the hepadnavirus family of liver-tropic DNA viruses that infect avian and mammalian species. The outer membrane-associated antigen -- HBsAg -- is responsible for eliciting a neutralizing protective antibody response. HBsAg is composed of three related proteins which share common amino acid sequences -- S,

preS2 + S, and PreS1 + PreS2 + S.

Antigen-containing compositions to be used in the present invention comprise PreS2 + S antigen of HBV and a metabolizable oil adjuvant. In one embodiment, the PreS2 + S antigen is recombinantly produced in Chinese Hamster Ovary cells. In a further preferred embodiment, the metabolizable oil adjuvant is the adjuvant MF59, described below.

In the methods according to the invention, the foregoing compositions are administered to a subject. Optionally, an anti-viral agent is administered before, at the same time as, or subsequent to the administration of the antigen-containing compositions. Administration of the antiviral agent before the administration of the antigen-containing compositions can range from one month to 24 months prior. There is a large population of infected hepatocytes in chronic HBV. Because liver injury results primarily from cellular immune responses to infected hepatocytes, it has been suggested that prolonged antiviral therapy precede other therapies to reduce the number of infected hepatocytes. (Genome, *J. Clin. Invest.*, 102(5):867-868, September, 1988.) Additionally, reducing the level of circulating HBsAg with antiviral agents can decrease the probability of deposition of antigen-antibody complexes upon the initiation of immunotherapy by decreasing the availability of free antigen.

An effective amount of the foregoing compositions is administered to a subject presenting with chronic HBV infection. As used herein, the term "effective amount," refers to the amount required to achieve an intended purpose for treatment without undesirable side effects, such as toxicity, irritation, or allergic response. Although individual needs may vary, the determination of optimal ranges for effective amounts of formulations is within the skill of the art. Human doses can also readily be extrapolated from animal studies (Katocs *et al.*, Chapter 27 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a formulation, which can be adjusted by one skilled in the art, will vary depending on several factors, including the age, health, physical condition, weight, type and extent

of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy, if required, and the nature and scope of the desired effect(s) (Nies *et al.*, Chapter 3 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996). A dosage in the range of about 5 to 100 µg is contemplated.

It is also contemplated that more than one administration of the compositions may be required. The time between administrations depends upon the number of administrations to be given. For example, if two administrations are given, the first can occur at zero months and the second can occur at one, two, or six months; if four administrations are given, they can occur at 0, 1, 2, and 6 months, respectively. Alternatively, the administrations can occur at monthly intervals. The time between multiple administrations can be readily determined by one skilled in the art. In one embodiment, eight administrations are given.

As used herein, the term "administering" includes, but is not limited to, transdermal, parenteral, subcutaneous, intra-muscular, oral, and topical delivery. A common requirement for any route of administration is efficient and easy delivery. In a preferred embodiment, the compositions are administered intra-muscularly.

The intended purpose of the methods of the disclosed invention is the amelioration of chronic HBV infection. Amelioration can be determined by, for example, a decrease in HBV DNA in blood, as measured using nucleic acid assays; a decrease in serum alanine aminotransferase (ALT) as measured by routine tests; or a transient flare in transaminases, as measured by routine tests. Preferably, the treatment according to the invention will result in the appearance of anti-HBe, with the concurrent decrease in/disappearance of HBe or, more preferably, the appearance of anti-HBs, with the concurrent decrease in/disappearance of HBsAg, all of which can be measured by routine immunoassays. For the purposes of this invention, normal ALT levels are defined as about 6 to 34 IU/L for females and about 6 to 43 IU/L for males.

As used herein, the term "metabolizable oil adjuvant" refers to adjuvants that comprise an oil that is non-toxic to and metabolizable by the subject to which it is

administered, preferably one of about 6 to about 30 carbon atoms including, but not limited to, alkanes, alkenes, alkynes, and their corresponding acids and alcohols, the ethers and esters thereof, and mixtures thereof. The oil can be any vegetable oil, fish oil, animal oil or synthetically prepared oil which can be metabolized by the body of the host animal to which the adjuvant will be administered and which is not toxic to the subject. The host animal is typically a mammal, and preferably a human. Mineral oil and similar toxic petroleum distillate oils are expressly excluded from this invention.

Exemplary sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like can also be used.

The technology for obtaining vegetable oils is well developed and well known. The compositions of these and other similar oils can be found in, for example, the Merck Index, and source materials on foods, nutrition and food technology.

The 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, can be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. These products are commercially available under the name NEOBEE® from PVO International, Inc., Chemical Specialties Division, 416 Division Street, Boongon, NJ, and others.

Oils from any animal source can also be employed in the adjuvants and immunogenic compositions of this invention. Animal oils and fats are usually solids at physiological temperatures due to the fact that they exist as triglycerides and have a higher degree of saturation than oils from fish or vegetables. However, fatty acids are obtainable from animal fats by partial or complete triglyceride saponification which provides the free fatty acids. Fats and oils from mammalian milk are metabolizable and can therefore be used in the practice of this invention. The procedures for

separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art.

Most fish contain metabolizable oils which can be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which can be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoid known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene which is particularly preferred herein. Squalane, the saturated analog to squalene, is also a particularly preferred oil. Fish oils, including squalene and squalane, are readily available from commercial sources or can be obtained by methods known in the art.

The oil component of these adjuvants and immunogenic compositions will be present in an amount from about 0.5% to about 20% by volume but preferably no more than about 15%, especially in an amount of about 1% to about 12%. It is most preferred to use from about 1% to about 4% oil.

Metabolizable oil adjuvants as contemplated herein are described in EP 0 399 843 B1, incorporated by reference. A preferred adjuvant is MF59, described therein and in Ott et al., "MF59, Design and Evaluation of a Sage and Potent Adjuvant for Human Vaccines," Chapter 10, pp. 277-296, *Vaccine Design: The Subunit and Adjuvant Approach*, Michael F. Powell and Mark J. Newman, eds., Plenum Press, New York, 1995, incorporated herein by reference. Specifically, MF59 is formulated as follows: 4.3 % w/v squalene, 0.5% w/v Tween 80® , 0.5% Span 85, and 400 μg/ml MTP-PE (N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-3(hydroxyphosphoryloxy)]ethylamide). MF59's mechanism of action appears to be the development of a strong $CD4^+$ T cell response.

The adjuvant can also comprise emulsifying and/or immunostimulatory agents. A substantial number of emulsifying and suspending agents are generally used in the pharmaceutical sciences. These include naturally derived materials such as gums from trees, vegetable protein, sugar-based polymers such as alginates and cellulose, and the like. Certain oxypolymers or polymers having a hydroxide or other

hydrophilic substituent on the carbon backbone have surfactant activity, for example, povidone, polyvinyl alcohol, and glycol ether-based mono- and poly-functional compounds. Long chain fatty-acid-derived compounds form a third substantial group of emulsifying and suspending agents which could be used in this invention. Any of the foregoing surfactants are useful so long as they are non-toxic.

Exemplary immunostimulatory agents include, for example, synthetic adjuvants that increase host immunity such as levamisole and isoprinosine. Levamisole is the levo isomer of tetramisole and potentiates humoral and cellular immunity through a T cell-dependent mechanism. Isoprinosine, a complex containing inosine, the purine precursor of adenosine and guanosine, promotes T cell mitogenesis. Tuftsin, a 4 amino acid peptide (Thr-Lys-Pro-Arg) homologous to a sequence in the immunoglobulin (Ig) heavy chain, primarily stimulates macrophages.

As used herein, the term "subject" refers to an animal, typically a mammal, preferably a human.

As used herein, the term "about" means ± about 10%.

As used herein, the terms "a", "an" and "the" refer to the singular and plural.

As used herein, "chronic HBV infection" generally refers to an HBV infection that has persisted beyond the acute stage. Chronic infection can result from chronic disease acquired either in childhood or as an adult. Chronic infection as a result of disease can generally be identified by the presence of HBsAg with abnormal levels of ALT, with or without detectable levels of HBeAg. Normal ALT levels generally range from 10 to 32 U/L (9-24 U/L for women, with the normal range for infants being twice that of adults). (Web page: *Focus : On Hepatitis C International*, "Laboratory Blood Tests," revised 7/10/98.) Chronic infection also includes, however, asymptomatic carriers that have normal ALTs; patients initially treated with anti-virals, such as interferon, to reduce virus below detectable levels; and post-liver transplant patients who are on either maintenance anti-viral therapy or receiving Ig infusions. The persistence of HBsAg for greater than 6 months following an acute infection is the hallmark of chronic infection. A defective antiviral T cell response has also been documented in patients with chronic infection (Boni et al., *J. Clin.*

Invest., 102(5):968-975, September, 1998).

As used herein, the term "anti-viral agent" or "anti-viral compound" includes, without limitation, interferon and nucleoside analogues, including lamivudine. Lamivudine has been used in reducing liver damage caused by chronic HBV and has been orally administered at doses of 25 mg - 300 mg per patient per day for periods of up to a year. The appropriate dosage of the anti-viral agent, however, will depend upon, *inter alia*, the agent and the patient.

A vaccine comprising recombinant HBV PreS2+S antigen (10 μg) and the metabolizable oil-in-water adjuvant MF59 has been shown to enhance immunogenicity in adults (Poland et al., 37th ICAAC, p. 216, September 28-October 1, 1997). After one dose of the vaccine, 89% of the test subjects developed seroprotective anti-HBs titers. One hundred percent of the test subjects were protected after two injections. Further, the geometric mean antibody titers of the PreS2+S/MF59 vaccine were over 100 times greater than those of a control vaccine containing aluminum as the adjuvant (Poland et al., *supra*). It was predicted by the present inventor that similar compositions can be used as immunotherapeutic agents for treating chronic HBV infection.

Example 1

A composition comprising recombinant PreS2+S in combination with the oil-in-water adjuvant MF59 was prepared. Recombinant PreS2+S was prepared as described below. All media and supplements were supplied by Hyclone.

The recombinant plasmid pKSVAdPreS-BglII was constructed by the insertion of the S + PreS2 gene using two adaptors (Figures 1-2). The cloning vector used in the construction of the recombinant expression system was pKSV-10 from Pharmacia. The pKSV-10 vector is7.2 Kb long and the cloning site is BglII. Viral DNA was isolated as described in Valenzuela et al., *Nature*, 280:815-819, August,30, 1979, incorporated herein by reference. An EcoRI/BglII fragment of about 1880 bps from the viral genomic DNA was partially digested with HhaI to isolate a 1230 bps EcoRI/HhaI fragment. Adaptors were designed to be ligated with EcoRI/HhaI

fragments to create BglII/BglII fragments for cloning into pKSV-10. pKSV-10/BglII was then ligated with Pre S BglII/BglII fragments to yield the DNA plasmid vector pKSVAdPreS-BglII. This was followed by transformation of competent cells HB101. Transformants were screened and maxi-preparations were made.

Cloning vector pSV7d/AML-dhfr was constructed by the insertion of the dhfr sequence into pSV7d (Stibbe et al., *Virology*, 123:436-442, 1982, incorporated herein by reference). DG44 (dhgr-) CHO cells (Urlaub et al., *PNAS USA*, 77:4216-4220, 1980, obtained at approximately passage number 100 in September, 1985 from Dr. Leslie Rall of Chiron) were co-transfected with pKSVAdPres-BglII and pAML/dhfr. Thereafter, 48 individual clones were grown in 24 well microwell plates. Clones were screened using the Abbott ELISA assay and a high producer clone, designated PKSVAdPreS 16, was amplified using methotrexate selection to yield, ultimately, clone PKSVAdPreS 16-12-80-30 (abbreviated HEP 30 cells). HEP 30 cells were expanded to prepare Master/Working Cell Banks, designated as HBV B30 cells.

Cells from the HBV B30 Master Cell bank were cloned and expanded in DME/F12 media supplemented with 6% dialyzed FBS and 50 μM methotrexate (MTX). After expansion, cells were adapted through several steps to MTX-DMEM/F12 medium, with a gradual reduction in FBS concentration from 6% to 2% in 3 L spinner flasks. Following adaptation, cells were inoculated into a 3 L stirred perfusion vessel and perfused with DMEM/F12, 2% FBS.

Some of the perfused cells were then subcloned in DMEM/F12 10% FBS media in the presence of 50μM MTX, and adapted to HBV production medium in the presence of 2% FBS and 2μM MTX through multiple shake flask passaging. At passage 14, a subclone was tested for productivity and frozen as parent culture vials (deposited to the Chiron Cell Culture Collection as CMCC11336). Some of these cells were thawed in DM122 medium and then expanded in DM122 medium supplemented with 200 mM L-glutamine, 1 mM (1:500) MTX. When cell density reached 4.0 - 8.0 x 10^5 viable cells/ml, the cells were concentrated to 1.0 x 10^9 in DM122, 10% human serum albumin (w/v) + 7.5 % (v/v) DMSO, and aliquoted at 1 ml per vial producing a new HBV Master Cell Bank (MBVMK001). These cells were

frozen at 1°C/minute to -96°C and then stored at ≤ -176°C.

MF59 was prepared as a sterile oil-in-water emulsion as described in Ott et al., *supra*, incorporated herein by reference. Ten µg of PreS2 + S in a 0.25 ml volume antigen was combined with 0.25 ml MF59 for a final dose volume of 0.5 ml. The vaccine components are listed in Tables 1 and 2 below.

Table 1
HBV Antigen Composition

Component	Amount Per Final Dose
PreS2 + S Antigen	0.02 mg
Sodium Chloride, USP	3.80 mg
Sodium Citrate, dihydrate, USP	2.15 mg
Citric Acid, monohydrate, USP	0.04 mg
Polysorbate 80 (Tween 80™)	0.01 mg
Water for Injection	Q.S.
pH range of 6.0 - 7.0	

Table 2
MF59 Adjuvant Composition

Component	Amount Per Final Dose
Squalene	9.75 mg
Polysorbate 80 (Tween 80™)	1.18 mg
Sorbitan Trioleate (Span 85™)	1.18 mg
Sodium Citrate, dihydrate, USP	2.15 mg
Citric Acid, monohydrate, USP	0.04 mg
Water for Injection	Q.S.
pH of 6.4	

Example 2

Five hundred microliters of the composition described in Example 1 were administered intra-muscularly into the deltoid of 13 human patients presenting with chronic HBV as determined by the presence of HBsAg for longer than six months. Eight patients were HBe positive. All 13 patients had elevated serum ALT (greater than 1.2 x ULN i.e., upper limits of normal), and had no evidence of decompensated liver disease. Administrations were performed at 0, 1, 2, and 6 months. Efficacy was determined by measuring decreases in HBV DNA, serum ALT levels, and decreases in HBeAg levels, as well as the appearance of anti-HBe antibodies one month after the second administration. HBV DNA was measured using the bDNA method (Chiron, and Chen et al., *J. Virol. Methods*, 53(1):131-7, May 1995, incorporated by reference herein in its entirety.) The levels of HBeAg, anti-HBe, HBsAg, and anti-HBs were measured using chemiluminescent immunoassays as follows. See also, U.S. Pat. No. 5,395,752, incorporated herein by reference in its entirety. Results are reported in optical density units (s/co).

For measuring HBeAg, 100 μl of serum was combined with 100 μl of human anti-HBe coupled to latex magnetic particles (600 μg/ml), and incubated for 18 minutes at 37 °C. One hundred μl of human anti-HBe coupled to DMAE (dimethyl acridinium ester, 300 30 million relative light units, "RLU", per ml) was added and incubated for 18 minutes at 37 °C. The particles were washed three times with buffer and then chemiluminscence measured. The buffer used had the following composition: 50 mM TRIS, pH 8.0; 500 mM potassium chloride; 0.09% sodium azide; 1 mM EDTA; 0.05% TWEEN 20; and 1.75% BSA, sulfhydryl modified and was filtered using a MILLIPAK-60 0.22 μm filter unit.

For measuring HBsAg, 100 μl of serum was diluted 1/100 in the buffer described above and combined with 100 μl of mouse anti-HBs (specifically binding to the anti-"a" neutralizing epitope of HBsAg) coupled to paramagnetic particles (for antibody concentrations of 280 μg/ml and 150 μg/ml, respectively) and 50 μl DMAE coupled to mouse anti-HBs (anti-"a", at 75.0 ng/ml) diluted in the buffer described above, and incubated for 18 minutes at 37 °C. The particles were washed three times

with buffer and then chemiluminscence measured.

For measuring anti-HBe, 50 μl of serum was combined with 100 μl of recombinant HBeAg SOD fusion protein (50 ng/ml) (see, for example, Choo, *et al.*, *Science*, 1989, *244*, 359-362, incorporated herein by reference in its entirety) and 100 μl of human anti-HBe coupled to latex magnetic particles (600 μg/ml), and incubated for 36 minutes at 37 °C. One hundred μl of human anti-HBe coupled to DMAE (300 x 10 E 6 RLU/ ml) was added and incubated for 18 minutes at 37 °C. The particles were washed three times with buffer and then chemiluminscence measured. The buffer used had the following composition: 100 mM borate buffer, pH 8.9; 0.09% sodium azide; 5 mM EDTA; 0.05% TWEEN 20; and 0.2% gelatin (fish) and was filtered using a MILLIPAK-60 0.22 μm filter unit.

Anti-HBs was measured using standard enzyme immunoassays (Abbott).

Significant reductions in HBeAg levels and development of anti-HBe antibodies in association with decreases in HBV DNA and serum ALT were elicited in 4 out of the 8 patients initially positive for HBe after 3-4 injections. Data for three patients are depicted in Figures 3a-b, Figures 4a-b, and Figures 5 a-b. Significant levels of anti-HBs antibodies were also elicited in several patients, including a patient that did not initially present with HBe. (See, for example, Tables 3 -5, Anti-HBs column). Two of the eight patients exhibited significant increases in serum ALT during the study. Both have resolved.

Upon further follow-up, over half of the 13 patients developed anti-HBsAg. Four patients serocoverted to anti-HBe, and 3 of the 4 seroconverted patients have become HBV DNA negative.

Example 3

Five hundred microliters of the composition described in Example 1, and 100 mg of lamivudine, are administered to a subject at time zero. Another 500 µl of the composition described in Example 1 and another 100 mg of lamivudine are administered at one month. Efficacy is determined by measuring anti-HBs titers as

well as assaying for HBs presence one month after the second administration. If detectable HBs is still present, a third administration is performed. This process is repeated until no more HBs is detected.

Example 4

One hundred mg of lamivudine is administered to a subject starting at time zero and administered on a continuous once daily dose schedule. Five hundred µl of the composition described in Example 1 are administered to a subject intra-muscularly at 1, 2, 3, and 7 month. Efficacy is determined by measuring HBeAg and anti-HBe titers as well as assaying for HBV DNA one month after the administration of the composition of Example 1. If detectable HBe is still present, a further administration of the composition of Example 1 is performed. If detectable HBe is present one month after this further administration, an additional administration is performed. This process is repeated until no more HBe is detected.

Example 5

Five hundred µl of the composition described in Example 1 are administered to a subject at time zero. One hundred mg of lamivudine are administered to a subject at one month. Efficacy is determined by measuring anti-HBs titers as well as assaying for HBs and HBe presence one month after the administration of the composition of Example 1. If detectable HBs or HBe is still present, a second administration of the composition of Example 1 is performed. If detectable HBs or HBe is still present, a third administration is performed. This process is repeated until no more HBs is detected.

Example 6

One hundred mg of lamivudine are administered daily for 6 months. Five hundred μl of the composition described in Example 1 are administered to a subject at months 7, 8, 9, and 13. If detectable HBs or HBe is present, additional administrations can be performed.

Example 7

Materials:

Chiron® HBV/MF59: Antigen: Recombinant HBV PreS2+S (20 µg/ dose) produced and purified from CHO cells. Adjuvant: MF59, a microfluidized oil-in-water emulsion. The antigen and MF59 were provided in separate vials to be mixed at the time of injection. Each dose was given intramuscularly in a total volume of 0.5 mL. The schedule consisted of four doses administered on a 0-, 1-, 2-, and 6-month schedule (Table 6).

Lamivudine (Epivir®, Glaxo Wellcome): Patients must have been treated for at least 1 month prior to initiation of Chiron® HBV/MF59 therapy. Lamivudine was continued for 7 months thereafter (i.e., one month after the fourth injection) at the dose level prior to Chiron® HBV/MF59 treatment (100 mg to 300 mg per day).

Measures of efficacy: At the conclusion of the study, the proportion of patients with each of the following parameters was/will be determined: loss of or reduction in HBsAg, loss of HBeAg, and anti-HBs or anti-HBe seroconversion. Changes in HBV DNA levels and serum ALT/ AST from pre-therapy with Chiron® HBV/MF59 will be summarized during and following the Chiron® HBV/MF59 treatment period. For this disclosure, available efficacy data were limited to selected timepoints and a full analysis has not been performed.

Methods:

A composition comprising recombinant PreS2+S antigens combined with MF59, an oil-in-water adjuvant, e.g. Chiron® HBV/MF59 (Table 2) was given to patients with chronic HBV infection receiving lamivudine. Subjects received four injections intramuscularly of Chiron® HBV/MF59 on a 0-, 1-, 2-, 6-month schedule. Patients continued treatment with lamivudine throughout the 6 month period of Chiron® HBV/MF59 injections and continued to receive lamivudine for one additional month after the fourth dose, after which lamivudine was discontinued.

Eligible subjects included patients with chronic HBV infection (defined by a history of positive HBsAg in serum for at least 6 months and positive HBsAg on the

screening examination) with compensated liver disease who were receiving lamivudine or were candidates for initiating therapy with lamivudine at screening. Subjects on lamivudine at the time of screening received lamivudine treatment for at least 1 month prior to the time of administration of the first Chiron® HBV/MF59 dose. These subjects continued lamivudine at their pre-study dose (between 100 to 300 mg per day). Subjects who were not receiving lamivudine treatment at the time of screening were treated for at least one month prior to initiating Chiron® HBV/MF59. These subjects received lamivudine at the approved dose of 100 mg per day. All patients with any evidence of poorly compensated or advanced liver disease based upon history, physical examination, clinical laboratory evaluation, or previous liver biopsy were to be excluded from participation.

Potential patients underwent an initial screening visit within 1 to 8 weeks prior to formal enrollment during which time they had a medical history taken and had a physical exam and blood and urine samples taken. Data were obtained at screening for the following serologic and virologic parameters of HBV infection (both qualitative and quantitative measures): HBsAg, HBeAg, anti-HBs, and anti-HBe. Additional assays included: liver function tests (LFTs): ALT, AST, alkaline phosphatase, bilirubin (total and direct), prothrombin time, total protein and albumin; complete blood count (CBC); and a urinalysis for protein, blood, and casts. Quantitative HBV DNA level was measured by polymerase chain reaction (PCR). Subjects were excluded if they had evidence of a lamivudine-resistant mutant as determined either prior to screening or at the time of screening (based upon elevated HBV DNA levels). Additional clinical laboratory tests at the screening visit included: anti-HCV antibody, anti-HIV antibody, and hepatitis delta antibody. Subjects who were positive for any of these were excluded from participation.

In addition to the inclusion and exclusion criteria, potentially eligible patients who were not receiving lamivudine therapy at the time of the study screening visit must have met the following additional inclusion criteria to establish eligibility: a serum ALT greater than 1.2 times the upper limit of normal, and more than 0.7 MEq/ml of HBV DNA, as measured by the bDNA assay. These laboratory values

were obtained from a blood sample collected at the screening visit.

Subjects who met all of the inclusion criteria and none of the exclusion criteria (including results from clinical laboratory testing) returned to receive their first intramuscular injection. They were observed for 30 minutes following each injection for evidence of immediate local and systemic reactions. They completed diary cards to describe local (e.g., pain, warmth, erythema, induration at the injection site) and systemic (e.g., fatigue, malaise, fever, chills, myalgia, arthralgia, nausea, headache, rash) reactions for 7 days post-injection. Diary cards were maintained by the subject to record medical problems and medications taken during the 7-day period. Subjects were contacted by telephone 7 days following each injection. If, at any time during the trial, a subject experienced any unusual, severe, or serious adverse event that may be therapy-related, they were instructed to be seen immediately in the clinic. Subjects were seen 14 days prior to the second, third, and fourth injections (i.e., at 1-month, 2-months, and 6-months, respectively) in order to have clinical laboratory tests (LFTs, urinalysis, creatinine) checked prior to receiving the next injection.

Seven months after the first injection of Chiron® HBV/MF59, lamivudine was stopped. Patients returned at Months 7.5, 8, 9, 10.5, and 12 for clinical and laboratory follow-up.

All adverse events (AEs) were monitored throughout the trial, from the initiation of Chiron® HBV/MF59 through the final visit. All prescription medications used throughout the trial were recorded

Number of subjects: The planned and actual enrollment was 24 subjects. All subjects received four injections of Chiron® HBV/MF59

Table 6
Days at which Injections Occurred

Immunization #1:	
# Immunized	24 (100%)
Immunization #2:	**Days Post 1st Immunization:**
Mean	31.7
Median	28.0
Std. Dev.	11.3
Min.	21
Max.	66
N	24
Immunization #3:	**Days Post 1st Immunization:**
Mean	60.7
Median	56.0
Std. Dev.	12.4
Min.	51
Max.	94
N	24
Immunization #3:	**Days Post 2nd Immunization:**
Mean	29.0
Median	28.0
Std. Dev.	3.2
Min.	23
Max.	39
N	24
Immunization #4:	**Days Post 1st Immunization:**
Mean	180.0
Median	180.5
Std. Dev.	9.1
Min.	168
Max.	198
N	24
Immunization #4:	**Days Post 2nd Immunization:**
Mean	148.3
Median	148.0
Std. Dev.	11.4
Min.	119
Max.	168
N	24
Immunization #4:	**Days Post 3rd Immunization:**
Mean	119.3
Median	120.0
Std. Dev.	11.5
Min.	

Table 7
Demography and Patient Characteristics at Screening.

Characteristic	Number of Patients (24 patients total)
HBsAg:	
Positive	24 (100%)
Anti-HBs:	
Negative	20 (83%)
Positive	4 (17%)
HBeAg:	
Negative	9 (38%)
Positive	14 (58%)
Other	1 (4%)
Anti-HBe:	
Negative	15 (65%)
Positive	8 (33%)
Other	1 (4%)
Hepatitis C:	
Negative	24 (100%)
Hepatitis Delta Infection:	
Negative	24 (100%)
HIV Infection:	
Non-Reactive	24 (100%)
ALT (U/L):	
Mean	69.5
Median	49.0
Std. Dev.	64.7
Min.	17
Max.	276
N	24

Summary of results

Subject Characteristics and Demography: The mean age of subjects was 42 years of age (range: 27 to 63 years). Out of the 24 subjects, 20 (83%) were male. Thirteen (54%) subjects were of Asian descent, 9 (38%) were Caucasian, one subject was African-American, and one subject was of mixed ethnicity. At the time of enrollment, 14 (58%) subjects were HBeAg positive, while 8 (33%) were anti-HBe positive (both EIA, Abbott); data were unavailable on one patient and another patient was negative for both. The mean serum ALT level was 70 IU/L (range: 17 IU/L to 276 IU/L) at the time of enrollment (Table 7). The mean duration of lamivudine use

(uninterrupted by more than 3 months) was 7.7 months (range: 1 to 33 months) prior to receiving the first injection of Chiron® HBV/MF59 (Table 7). Sixteen of the 24 patients had received treatments in addition to or other than lamivudine prior to entering this trial. These treatments included interferon (including the intron-a and CIFN forms), Famciclovir, and a Hepatitis B vaccine (CV-1899) (Table 9).

As of the January 1, 2000, all 24 subjects had received all four injections of Chiron® HBV/MF59. Twenty-two patients have been followed for a minimum of two months post discontinuation of lamivudine. Among these 22 patients data were collected up to Month 10.5 in five patients, while two patients had completed the study entirely (Month 12).

Table 8
Lamivudine Dosing

Duration of Lamivudine Prior to Enrollment:	**Months**
Mean	7.67
Median	5.35
Std. Dev.	7.78
Min.	0.9
Max.	33.3
N	24
Total Duration of Lamivudine Use:	**Months**
Mean	14.46
Median	12.25
Std. Dev.	7.72
Min.	7.6
Max.	40.3
N	24

Table 9
Pre-Trial Hepatitis Treatments Other than Lamivudine

Treatment	**Patients**
Interferon	101, 601, 602
Interferon Alfa (intron-a)	201, 203, 204, 208, 401, 402, 403, 404, 501
Interferon (CIFN (consensus interferon))	202
Famciclovir	102, 602
Hepatitis B Vaccine (CV-1899)	101, 207

Clinical laboratory evaluation: Historical information on pre-study serum ALT levels were collected as extensively as possible. The period and number of available data varied for each subject. Many subjects had widely fluctuating levels of ALT over variable time periods including substantial elevations of ALT levels pre-study prior to receiving lamivudine. Among the 24 subjects, nine had documented evidence of pre-study hepatitis flares with peak serum ALT levels over 700 IU/L. The median peak ALT level among these nine patients was 1035 IU/L with a range of 782 IU/L to 3116 IU/L.

Serum ALT levels were either normal or minimally elevated (< 2 times upper limit of normal) in most subjects both at the time of screening and during the first seven months of study participation. This would be expected since, during this period, all subjects were receiving lamivudine. In two patients (205 and 601), the serum ALT levels were persistently abnormal, with the maximum recorded level on-study while receiving lamivudine being 112 IU/L and 119 IU/L respectively. Both patients had HBV DNA detectable by bDNA assay while receiving lamivudine, and patient 601 showed a marked increase in the level of HBV DNA while receiving lamivudine, suggesting the possibility of the development of lamivudine-resistant mutant.

The data on serum ALT levels are summarized in Tables 10 and 11. Table 10 shows that maximum ALT level after lamivudine discontinuation for the 22 of the 24 patients for whom data were available up to the most recent visit. In 10 patients, maximum ALT values were less than 50 IU/L. Six patients had maximum ALT values between 50 IU/L to 100 IU/L, 5 patients had maximum values between 101 IU/L to 200 IU/L, and a single patient (502) had a peak ALT value >200 IU/L, with a peak level of 1870 IU/L. Among the 12 patients who had maximum ALT levels greater than 50 IU/L, the maximum ALT value after discontinuation of lamivudine was compared with the maximum historic value documented before enrolling in this study (Table 11). With the exception of a single patient (502), all patients had maximum ALT levels after lamivudine discontinuation that were either lower than (10

out of 22 patients) or comparable to (1 out of 22 patients) the highest pre-study values documented.

Patient 502 exhibited an early virologic relapse upon the discontinuance of lamivudine that was resolved by 10.5 months. One month after discontinuing lamivudine (Month 8), the HBV DNA level had increased to 511 Meq/mL and the ALT level increased to 63 IU/L, indicating an early virologic relapse was taking place. Two months after discontinuing the lamivudine treatments (month 9), the ALT level of patient 502 peaked at 1870 IU/L, however the HBV DNA level had decreased to 118 mEq/ml. After month 9, the ALT levels of patient 502 decreased steadily to 46 IU/L at month 10.5.

Table 10
Maximum Alt Level During Period after Discontinuation of Lamivudine.

Maximum ALT Level (during period after discontinuation of lamivudine)	Number of Subjects (N=22)	Subject ID No.
≤ 50 IU/L	10	101, 102, 201, 202, 203, 207, 403, 404, 505, 602
51 IU/L to 100 IU/L	6	204, 206, 401, 501, 503, 601
101 IU/L to 200 IU/L	5	205, 402, 504, 603, 604
> 200 IU/L	1	502

Table 11
Maximum Pre-study ALT Level and Maximum Post-lamivudine ALT Level in Subjects Whose Maximum Post-lamivudine ALT Level Was Greater than 50 IU/L.

Patient ID Number (N=12)	Maximum Pre-study Serum ALT level (IU/L)	Maximum serum ALT Level During Post-Lamivudine Follow-up (IU/L)
204	342	54
205	141	144
206	271	52
401	328	77
402	400	125
501	112	57
502	810	1870
503	62	53
504	924	167
601	339	69
603	299	130
604	285	155

Quantitative HBV DNA evaluation: Data on quantitative HBV DNA levels are available for a limited number of time points. Three subjects (402, 504, and 601) had significant increases (>100-fold) in HBV DNA while on lamivudine. The likely reason for these increases is the emergence of HBV escape mutants with resistance to lamivudine. HBV genotyping of these samples will be performed. Early information on viral relapse following discontinuation of lamivudine were available in 16 patients who had HBV DNA levels measured at Month 8 or 9. Among these 16 patients, seven (401, 403, 404, 501, 503, 504, 505) had HBV DNA levels below the level of detection of the bDNA assay at Month 8 or 9 (one or two months after lamivudine discontinued). This includes one patient (504) who may have developed an escape mutant while on lamivudine (with a HBV DNA level 577 MEq/mL at Month 7) but who subsequently became HBV DNA negative during follow-up. The remaining nine patients in whom HBV DNA was measured had detectable HBV DNA after lamivudine was discontinued.

Table 12
Maximum Pre-study and Maximum Post-Final Injection HBeAg and Anti-HBe Levels

	Pre-Study Maximum Levels		Post-Study Maximum Levels	
Patient	HBeAg (IU/mL)	Anti-HBe (IU/mL)	HBeAg (IU/mL)	Anti-HBe (IU/mL)
101	16.4	1.6	30.2	1.8
102	0.2	9.9	<0.1	46.4
201	<0.1	41.5	<0.1	47.0
202	<0.1	36.9	<0.1	24.2
203	<0.1	5.2	<0.1	5.7
204	1.0	4.5	1.9	3.2
205	<0.1	122.6	<0.1	137.3
206	1.9	5.7	18.4	2.7
207	9.7	2.9	18.9	2.4
208	3.3	4.6	17.8	2.5
401	<0.1	44.1	<0.1	68.8
402	0.2	4.1	0.4	4.2
403	<0.1	419.7	<0.1	355.5
404	1.2	5.0	<0.1	4.5
501	<0.1	261.9	<0.1	338.2
502	1.4	4.3	13.0	2.8
503	0.2	62.1	1.1	17.1
504	1.9	3.8	3.8	2372.0
505	3.1	4.4	<0.1	30.7
601	14.7	1.8	17.6	1.8
602	2.9	4.3	<0.1	5.2
603	<0.1	274.2	<0.1	162.0
604	<0.1	165.1	<0.1	205.5

Quantitative HBeAg and anti-HBe evaluation: Limited data on quantitative changes in HBeAg and anti-HBe levels were evaluated. HBeAg and anti-HBe titers were measured by an in-house EIA. The lower limit of detection of the in-house HBeAg assay is comparable to a commercial EIA (Abbott). Among the 14 patients who were HBeAg positive at screening (i.e. > 0.1), 4 subjects (102, 404, 505, and 602) had quantitative HBeAg below the level of detection after the final injection. Two of these four patients (102 and 505) have also had significant (>4-fold) increases in anti-HBe titer (Table 12).

The foregoing examples are meant to illustrate the invention and not to limit it in any way. Those skilled in the art will recognize modifications within the spirit and scope of the invention as exemplified in the appended claims.

All references cited herein are hereby incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for treating chronic Hepatitis B virus (HBV) infection, said method comprising administering an effective amount of a composition comprising PreS2 + S antigen and a metabolizable oil adjuvant to a subject.

2. The method of claim 1, wherein the PreS2 + S antigen is administered at from about 5 to about 100 μg per dose.

3. The method of claim 2, wherein the PreS2 + S antigen is administered at about 20 μg per dose.

4. The method of claim 1, wherein the PreS2 + S antigen is administered more than one time.

5. The method of claim 4, wherein the PreS2 + S antigen is administered eight times.

6. The method of claim 4, wherein the PreS2 + S antigen is administered four times.

7. The method of claim 4, wherein the PreS2 + S antigen is administered until the HBV infection is ameliorated.

8. The method of claim 4, wherein the PreS2 + S antigen is administered until the HBV infection is eliminated.

9. The method of claim 7, wherein the amelioration of the HBV infection is determined by measuring a decrease in the concentration of a serum component selected from the group consisting of HBV DNA, alanine aminotransferase (ALT), HBsAg and HBeAg.

* C L M S *

10. The method of claim 7, wherein the amelioration of the HBV infection is determined by measuring an increase in the concentration of a serum component selected from the group consisting of anti-HBs and anti-HBe.

11. The method of claim 9, wherein the amelioration of the HBV infection is determined by measuring a decrease in the concentration of serum ALT to normal levels.

12. The method of claim 1 wherein said PreS2 + S antigen is recombinantly produced in Chinese Hamster Ovary cells.

13. The method of claim 1 wherein said metabolizable oil adjuvant is MF59.

14. The method of claim 1 further comprising administering an anti-viral agent or compound.

15. The method of claim 14, wherein the anti-viral agent is lamivudine.

16. The method of claim 15 wherein the lamivudine is administered daily at from about 100 to about 300 mg per day.

17. The method of claim 16, wherein the lamivudine is administered daily at about 100 mg per day.

18. The method of claim 14, wherein the anti-viral agent is administered for at least one month prior to administering the Pre-S2 + S antigen.

19. A kit for treating chronic Hepatitis B infection comprising a composition comprising recombinant PreS2 + S antigen, said kit further comprising an anti-viral agent or compound, and a composition comprising a metabolizable oil adjuvant.



20. The kit of claim 20 wherein the metabolizable oil adjuvant is MF59.

21. The kit of claim 19 or 20 wherein the recombinant PreS2 + S antigen is produced in Chinese Hamster Ovary cells.

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