Title: DIAGNOSTIC AND THERAPEUTIC METHODS AND AGENTS

Abstract: The present invention relates generally to the fields of therapy and diagnosis of Hepatitis B virus (HBV) infection in animal species including humans. The present invention further provides compounds and compositions useful in the treatment of HBV infection in animal species such as humans including agents which facilitate clearance of HBV and in particular chronic HBV infection.
BACKGROUND OF THE INVENTION

5 FIELD OF THE INVENTION

The present invention relates generally to the fields of therapy and diagnosis of Hepatitis B virus (HBV) infection in animal species including humans. The present invention further provides compounds and compositions useful in the treatment of HBV infection in animal species such as humans including agents which facilitate clearance of HBV, and, in particular, chronic HBV infection.

DESCRIPTION OF THE PRIOR ART

Bibliographic details of the publications referred to in this specification are also collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Hepatitis B virus (HBV) causes debilitating disease conditions and can lead to acute liver failure or chronic infection that can also lead to severe liver damage, hepatocellular carcinoma and liver failure. HBV is a DNA virus which replicates via an RNA intermediate and utilizes reverse transcription in its replication strategy. The HBV genome is of a complex nature having a partially double-stranded DNA structure with overlapping open reading frames encoding envelope, pre-core, core, polymerase and X genes.

The polymerase gene is the largest open reading frame and it encodes for the multi-functional polymerase (Pol) protein. The polymerase gene overlaps all six other genes including the core gene that encodes for HBc and the precore gene that encodes for the hepatitis Be antigen (HBeAg), the three envelope genes PreSI, PreS2 and S that encodes for the large, middle and small envelope proteins respectively (LHBs, MHBs and SHBs) The small envelope protein is also referred to as the hepatitis B surface antigen (HBsAg). The X gene encoding for the multifunctional X protein.
To understand the special populations with chronic HBV infection one must understand the natural history of HBV infection. With up to 30% of patients with chronic HBV infection developing cirrhosis and or liver cancer, the course of HBV must be defined individually for each patient being evaluated for clinical trials or treatment. HBeAg negative chronic hepatitis currently represents the predominant form of chronic hepatitis due to HBV in several parts of the world where non-genotype A infection is common such as in Africa, Asia, the Middle-East, the Mediterranean Basin and South America.

The important spontaneous seroconversion from HBeAg to anti-HBeAg antibodies (and a concomitant decrease in HBV DNA levels) occurs in 1 to 10% of chronic hepatitis B carriers (in patients with wild type virus) per annum, but seroconversion from HBsAg to anti-HBsAg antibodies with clearance of HBV from the liver, is very uncommon (at or less than 1% per year). Thus, diagnostics which monitor parameters involved in HBsAg clearance and treatments which lead to HBsAg clearance will play an important role in the monitoring and treatment of patients with chronic hepatitis B.

Chronic HBV infection is defined as the persistence of HBsAg for more than six months. HBV persistence may be due to the stable nature of covalently closed circular (cccDNA), infection of immunologically privileged sites and/or HBV-specific immune suppression., 1998). Overall, there is a reduction in functional HBV-specific CD4+ and CD8+ T-cell in persistent HBV infection when compared with individuals who successfully clear infection. In individuals with persistent HBV infection, the HBV-specific CD8+ T-cell response is significantly diminished when evaluated by proliferative responses to whole HBV antigens or defined epitopes in HLA-A2 positive chronic carriers (Ferrari et al, J Immunol. 145:3442-9, 1990; Maini et al, J Exp Med 191:1269-80, 2000).

The host virus relationship is a dynamic process in which many viruses such as HBV attempt to maximize their invisibility while the host attempts to prevent and eradicate infection. Initially, a virus must bind and enter a target cell and migrate to the appropriate cellular compartment in order to replicate and infect other cells. Infected cells may be triggered by the virus to produce cytokines (e.g. TNF-α and IFN-γ) that inhibit one or more stages of the viral replication cycle, thereby limiting the extent of the infection.

Host monocytes and macrophages play a key role in the early response to the virus as they
secrete pro-inflammatory cytokines, such as IL-1, TNF-α, IL-6, IL-12 and IL-18 that have indirect and direct effects on the infection. They can recruit further monocytes, natural killer (NK) cells and T-cells to perform functions and they can also help switch to the appropriate Th function to help eradicate the virus.

Innate immunity to microbial pathogens, leading to the production of these pro-inflammatory cytokines, occurs as a result of the activation of Toll Like Receptors (TLRs). TLRs have been identified as a major class of pattern-recognition receptors. The role of TLRs involving bacterial products, e.g. endotoxin and peptidoglycan has recently been clarified (Akashi et al, J Immunol. 164: 3471-3475, 2000; Takeuchi et al, Immunity, 11: 443-451, 1999; Tapping et al, J Immunol. 165: 5780-5787, 2000). More than 13 TLRs have been identified and they play an important role in activation by a number of different bacteria. Recently, this has been extended to viruses with the demonstration that respiratory syncytial virus (RSV) stimulates TLR-4 in a murine model (Kurt-Jones et al., Nat Immunol. 1: 398-401, 2000; Haeberle et al, J Infect Dis. 186: 1199-1206, 2002). In addition, Measles Virus (MV) has been shown to activate TLR-2 dependent signals (Bieback et al, J Virol. 76: 8729-8736, 2002) and double-stranded RNA (the core of many viruses) has been shown to directly mediate responses to through TLR-3 (Matsumoto et al, Biochem Biophys Res Commun. 293: 1364-1369, 2002). TLR7 and TLR9, both located in the endosomal membrane. TLR7 and TLR8 recognize viral single-stranded RNA as well as imidazoquinolines such as imiquimod and resiquimod and guanosine analogs. TLR9 recognizes bacterial or viral DNA, including synthetic CpG oligodeoxynucleotides.

Stimulation of TLRs by their ligands initiates the activation of complex networks of intracellular signal transduction pathways to coordinate the ensuing inflammatory response. Important components of these signaling networks are the adaptor protein MyD88 (and related proteins), several protein kinases (including IRAK-I, p38 MAP kinase and IKK kinase), TRAF6 and the transcription factor NF-κB. Activation of NF-κB leads to the expression of a variety of pro-inflammatory mediators (e.g. TNFα, IL-1, IL-6 and MCP-I) (Akira, S. J Biol Chem 278, 38105-8; 2003; Barton, G. M. & Medzhitov, R. Science 300, 1524-5 2003; Beutler, B., et al, J Leukoc Biol 74, 479-85; 2003). The activation of adaptive immune response by TLR7 is mediated by MyD88-dependent pathway. TLR7, TLR8, and TLR9 induce interferon (IFN)-alpha in cells such as plasmacytoid dendritic cells (pDCs). This induction requires the formation of a complex consisting of the adaptor MyD88, tumor
necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and IFN regulatory factor (IRF) 7. TLR3 and TLR4 are also capable of signaling via MyD88-independent pathways, involving the adaptor molecules TRIF (for TLR3 and 4) and TRAM (for TLR4) [Lien, E. & Golenbock, D. T. Nat Immunol 4, 1162-4, 2003].

The signals induced upon TLR activation in turn control the activation of the specific immune response. There is evidence that the specific immune system only responds to a pathogen after it has been recognized and processed by the innate immune system. T-cell receptors require co-stimulatory molecules, such as CD80 and CD86, to be expressed on the surface of the antigen-presenting cell in association with the peptide-MHC complex in order for activation to occur. The expression of these co-stimulatory molecules is controlled in part by TLRs (Pasare, C. & Medzhitov, R. Curr Opin Immunol 15, 677-82, 2003). They are also important in activating B-cells to produce rheumatoid factors (Leadbetter, E. A. et al. Nature 416, 603-7 2002).

There is a need to investigate the role of pathogen-mediated down-regulation of TLRs and to develop mechanisms to combat infection by assisting the innate immunity system.

SUMMARY OF THE INVENTION

The present invention identifies that up-regulation of Toll-like receptors and in particular TLR7 or another component of the TLR7-signaling pathway is associated with clearance of HBV from infected subjects and hence represent useful targets to treat chronic HBV infection. More particularly, the present invention identifies that up-regulation of TLR7 is required for HBsAg clearance and is associated with the generation of anti-HBsAg antibodies and resolution of HBV infection.

TLR7 is, therefore, a useful target for therapeutic agents to treat subjects such as humans infected with HBV. The therapeutic agents up-regulate TLR7 and promote a decrease in HBsAg and the generation of anti-HBsAg antibodies which assist in the clearance and resolution of HBV infection. TLR7 is also a useful diagnostic target to monitor therapeutic protocols and hence TLR7 is useful as an epidemiological management tool.

The present invention provides, therefore, therapeutic agents capable of up-regulating levels or activity of TLR7 or a component in the TLR7-signaling pathway. Such agents may be
used alone or in combination with other anti-HBV treatment protocols including nucleoside or nucleotide analogs, antiviral and/or immunomodulatory agents such as interferon (IFN) and vaccine compositions capable of stimulating a cellular and/or humoral response against HBV or its components.

In particular, the present invention contemplates a method for monitoring infection by HBV during or subsequent to treatment in a subject, said method comprising determining changes in levels of TLR7 or a component in the TLR7 signaling pathway during or subsequent to treatment.

More particularly, the present invention provides a method for monitoring acute, chronic or persistent infection by HBV during or subsequent to treatment in a subject, said method comprising determining changes in levels of TLR7 or a component in the TLR7-signaling pathway during or subsequent to treatment wherein elevated levels of TLR7 is indicative of improved potential for HBsAG clearance and resolution of HBV infection.

TLR7 may be measured in any cells such as but not limited to Kupffer cells, liver cells and/or peripheral monocytes.

The present invention contemplates, therefore, therapeutic and diagnostic agents and compositions including vaccine compositions comprising same useful in the treatment and/or diagnosis of infection by HBV or its clearance during or subsequent to treatment.

The present invention also provides a method of treating a subject infected with HBV, said method comprising administering to said subject an effective amount of an agent including a composition comprising an agent which up-regulates the level of TLR7. Reference to an agent includes a single agent or two or more agents. Another agent, which is an anti-HBV agent such as a nucleoside or nucleotide analog or antiviral and/or immunomodulatory agents such as interferon (IFN), may also be in the composition or administered prior to, simultaneously with or subsequent to administration of the TLR7-up-regulating agent. Reference to a composition includes a vaccine or vaccine composition or medicament.

The present invention contemplates a method for monitoring a response to a therapeutic protocol directed against infection by HBV said method comprising determining the level or activity of TLR7 wherein the level of TLR7 or a component within the TLR7-signaling
pathway is indicative of the clearing or resolving capacity of the therapeutic protocol.

Preferred subjects are mammals or avian species. Most preferred subjects are humans. Animal models are also contemplated by the present invention.

A summary of sequence identifiers used throughout the subject specification is provided in Table 1 and in Figure 16.

### TABLE 1

**Summary of sequence identifiers**

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<tr>
<td>2</td>
<td>Probe oligonucleotide primer for TLR1</td>
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<tr>
<td>53</td>
<td>Reverse oligonucleotide primer for HBV RNA</td>
</tr>
</tbody>
</table>
BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation of the time line of treatment with a pegylated IFN and either oral placebo or lamivudine.

Figure 2 is a graphical representation showing TLR3, 5, 7 and 9 expression in PBMC.

Figure 3 is a graphical representation showing TLR7 expression following treatment with pegylated IFN.

Figure 4 is a graphical representation showing (A) Longitudinal TLR7 mRNA expression in the T-cells in HBsAg clearance, HBeAg seroconversion and nonresponder to pegylated interferon alfa-2a; (B) cycle threshold of GADPH; (C) HBcAg specific interferon-\(\gamma\) producing CD4+ T cells in HBsAg clearance and HBeAg seroconversion; (D) HBsAg specific interferon-\(\gamma\) producing CD4+ T cells in HBsAg clearance and with HBeAg seroconversion; (E) HBV Core peptide 18-27 specific interferon-\(\gamma\) producing CD8+ T cells in HBsAg clearance and HBeAg seroconversion; and (F) the characteristics of a HLA-A2 positive patient with HBsAg clearance.

Figure 5 is a graphical representation showing a dynamic time course suppression of TLR7 expression in HBsAg + patients compared to anti-HBc+/anti-HBs+ in co-culture model.

Figure 6 is a graphical representation showing the effects of a TLR7 agonist on (A) expression of TLR7; (i) TLR7 levels based on real time PCR; (ii) TLR levels based on Western Blot and (B) MTT assay.

Figure 7 is a graphical representation showing HBV RNA levels after co-culture with HBsAg or HBsAg + TLR relative to controls.
**Figure 8** is a photographic representation showing specificity of HBA RNA detection.

**Figure 9** is a graphical representation showing cccDNA in PBMCs after co-culture with HBV.

**Figure 10** is a graphical representation showing (A) CD4 responsive as (B) CDS responsive with respect to the number of IFN-γ-positive lymphocytes.

**Figure 11** is a graphical representation showing HBV DNA in co-culture in the presence or absence of HBsAg and/or TLR7.

**Figure 12** is a graphical representation showing pathways up-regulated at mRNA level on Day 7 of co-culture.

**Figure 13** is a graphical representation showing (A) TLR7 mRNA expression on T-cells of patient with natural immunity to HBV (HBV immune) and HBsAg plus HBeAg positive patients with serum ALT more than 2 times the upper limit of normal (HBV Positive) after incubation wild-type HBV in the presence of autologous antigen presenting cells; (B) A real time PCR for TLR7 RNAi showing potent silencing of TLR7 with no effect on GADPH; (C) HBeAg specific interferon-γ producing CD4+ T-cells in the *in vitro* model; and (D) HBV Core peptide 18-27 specific interferon-γ producing CD8+ T cells in the *in vitro* model.

**Figure 14** is a graphical representation showing (A) HBeAg specific interferon-γ producing CD4+ T-cells in the HBV positive group after treatment with different doses of TLR7 agonist; and (B) HBV Core peptide 18-27 specific interferon-γ producing CD8+ T cells in the HBV positive group after treatment with different doses of TLR7 agonist.

**Figure 15.** is a graphical representation showing (A) the enhancement in interferon-γ release from CD4+ T-cells of HBV Positive patients by TLR7 agonist 2.0 ng/ml in *in vitro* culture was significantly reduced when both interferon-α and interferon-β Ab was added into the *in vitro* culture. The addition of either interferon-α Ab or interferon-β Ab independently into the *in vitro* culture did not result in a significant reduction in the interferon-γ release from CD4+ T-cells of HBV Positive patients by TLR7 agonist 2.0 ng/ml; and (B) TLR7 agonist can
stimulated enhanced type I interferon (interferon-α/β) secretion by monocytes matured dendritic cells.

**Figure 16** is a representation showing oligonucleotide primers and probes used for Taqman analysis.

**DETAILED DESCRIPTION OF THE INVENTION**

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The present invention is predicated in part on the determination that up-regulation of a TLR and in particular TLR7, in at least liver cells, Kupffer cells and peripheral blood monocytes, dendritic cells or other antigen presenting cells and T-cells facilitates resolution of HBV infection and clearance of HBsAg. The elevation of levels of TLR7 is proposed, therefore, to enhance both the innate immune response and the HBV specific immune response *via inter alia* generation of anti-HBsAg antibodies. The presence, absence, levels or activity of TLR7 provides, therefore, the focus for the development of therapeutic and diagnostic protocols for HBV infection or for monitoring the infection before, during or after therapeutic intervention.

The present invention is particularly relevant for subjects with chronic HBV infection.

The present invention provides, therefore, agents which up-regulate TLR7 or a component of the TLR7 signaling pathway and methods for the treatment of HBV infection. Compositions such as vaccine compositions useful in generating a cellular or humoral response to HBV and/or its components is also contemplated by the present invention when used in combination with a TLR7-upregulating agent or agents. The agents of the present invention may be in the form of a composition. When the composition also comprises an antigen to induce an immune response then the composition may also be called a vaccine composition. The TLR7-upregulating agents or compositions may be included used together with the vaccine composition or maintained separately. The compositions may, therefore, comprise a single agent or multiple TLR7-modulating agents or one or more TLR7-modulating agents...
and one or more anti-HBV agents such as a nucleoside or nucleotide analog or antiviral
and/or immunomodulatory agents such as interferon (IFN) and/or an antigen which can
stimulate an immune response against HBV and/or its components. In addition, multiple
compositions may be employed given simultaneously or sequentially wherein each
composition comprises a single agent or single group of agents, which are selected from a
TLR7-modulating agent, an antibody, a nucleotide or nucleoside analog or other anti-HBV
agent or antiviral and/or immunomodulatory agents such as interferon (IFN) or an antigen
useful in a vaccine composition.

The present invention further contemplates a method for monitoring a response to a
therapeutic protocol as well as a means for determining the efficacy of a therapeutic regimen.
In particular, the present invention provides a clinical or epidemiological management tool
for HBV infection in animals, such as mammals and in particular humans. The method of
this aspect of the present invention is directed to determining levels of TLR7 or monitoring
TLR7 levels or activity over time during or subsequent to treatment.

Accordingly, one aspect of the present invention contemplates a method for monitoring acute,
chronic or persistent infection by HBV in a subject said method comprising determining the
levels or activity of TLR7 or a component in the TLR7 signaling pathway wherein the level
or activity of TLR7 is indicative of the capacity of the subject to induce clearance of HBsAg,
to generate anti-HBsAg antibodies and to facilitate resolution of HBV infection.

Another aspect of the present invention is directed to a method for monitoring acute, chronic
or persistent infection by HBV during or subsequent to treatment in a subject, said method
comprising determining changes in levels of TLR7 or a component in the TLR7-signaling
pathway during or subsequent to treatment wherein elevated levels of TLR7 is indicative of
improved potential for HBsAG clearance and resolution of HBV infection.

A related embodiment of the present invention provides a method for monitoring a response
to a therapeutic protocol directed against infection by HBV in a subject said method
comprising determining the level or activity of a TLR7 or a component of the TLR7 signaling
pathway wherein the level or activity of TLR7 or component of the TLR7 signaling pathway
is indicative of the capacity of the subject to resolve HBV infection.

Still yet another aspect of the present invention is directed to method of treating a subject
infected with HBV said method comprising administering to said subject an effective amount of an agent which up-regulates TLR7 or a component of the TLR7 signaling pathway.

The latter embodiment includes providing the subject with a TLR7-up-regulating agent together with other forms of anti-HBV therapy such as a nucleoside or nucleotide or antibody or other anti-HBV agent or antiviral and/or immunomodulatory agents such as interferon (IFN) or an antigen useful in a vaccine composition. The combination therapy may be in the form of a single composition or multiple compositions given simultaneously or sequentially.

It is to be understood that unless otherwise indicated, the subject invention is not limited to specific formulations of components, manufacturing methods, dosage regimens, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a compound" includes a single compound, as well as two or more compounds; reference to "an agent" includes a single agent, as well as two or more agents; reference to the HBV includes a single strain or variant of HBV or two or more strains of variants or HBV; and so forth.

In describing and claiming the present invention, the following terminology is used in accordance with the definitions set forth below.

The terms "compound", "active agent", "pharmacologically active agent", "medicament", "active" and "drug" are used interchangeably herein to refer to a chemical compound that induces a desired pharmacological and/or physiological effect. The terms also encompass pharmaceutically acceptable and pharmacologically active ingredients of those active agents specifically mentioned herein including but not limited to salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the terms "compound", "active agent", "pharmacologically active agent", "medicament", "active" and "drug" are used, then it is to be understood that this includes the active agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, metabolites, analogs, etc. The term "compound" is not to be construed as a chemical compound only but extends to peptides, polypeptides and proteins as well as genetic molecules such as RNA, DNA and chemical
analogs thereof. Reference to a "peptide", "polypeptide" or "protein" includes molecules with a polysaccharide or lipopolysaccharide component. The term "agonist" or "potentiator" is an example of a compound, active agent, pharmacologically active agent, medicament, active and drug which up-regulates the level of TLR7 or a component in the TLR7 signaling pathway.

The present invention extends to compositions comprising combinations of compounds or agents such as an agonist of TLR7 or a component in the TLR7 signaling pathway and a nucleoside or nucleotide analog or anti-HBsAg antibody, or antiviral cytokines (eg., IFN-oc., IFN-γ, IL-2, TNF-oc) or other immunomodulatory or anti-HBV agents.

The present invention contemplates, therefore, compounds useful in up-regulating levels of TLR7 or potentiating general or specific TLR7 signaling. It is proposed that up-regulating TLR7 signaling facilitates clearance of HBsAg and anti-HBsAg antibody production. This is proposed to lead to resolution of HBV infection. The compounds have an effect on acute, chronic infection by HBV. The cells carrying TLR7 to be modulated include inter alia liver cells, Kupffer cells and peripheral/monocytes. A liver cell includes a hepatocyte. Reference to a "compound", "active agent", "pharmacologically active agent", "medicament", "active" and "drug" includes combinations of two or more actives such as an agonist or potentiator of TLR7 or TLR7 signaling. A "combination" also includes multi-part such as a two-part pharmaceutical composition where the agents are provided separately and given or dispensed separately or admixed together prior to dispensation.

For example, a multi-part pharmaceutical pack may have a modulator of TLR7 and one or more anti-viral agents. The terms "modulating" or its derivatives, such as "modulate" or "modulation", are used to describe up- or down-regulation. In relation to TLR7, the aim is to up-regulate the TLR7 levels or activity to assist in HBsAg clearance.

Reference to "TLR7" includes a component of TLR7 signaling pathway.

The terms "effective amount" and "therapeutically effective amount" of an agent as used herein mean a sufficient amount of the agent to provide the desired therapeutic or physiological effect. Furthermore, an effective TLR7-modulating amount of an agent is a sufficient amount of the agent to directly or indirectly up-regulate the function or level of
TLR7 or to potentiate TLR7 signaling. This may be accomplished, for example, by the agents acting as an agonist (i.e. a potentiator) of the TLR7 or its signaling components such as agents which are, or mimic, components of the TLR7 signaling pathway, and which induce the TLR7 signaling pathway via other cellular receptors or by the agents antagonizing inhibitors of TLR7 signaling components. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what is an appropriate "effective amount". The exact amount required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation.

By "pharmaceutically acceptable" carrier, excipient or diluent is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e. the material may be administered to a subject along with the selected active agent without causing any or a substantial adverse reaction. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emulsifying agents, pH buffering agents, preservatives, and the like. A pharmaceutical composition may also be described depending on the formulation as a vaccine composition.

Similarly, a "pharmacologically acceptable" salt, ester, emide, prodrug or derivative of a compound as provided herein is a salt, ester, amide, prodrug or derivative that this not biologically or otherwise undesirable.

The terms "treating" and "treatment" as used herein refer to reduction in severity and/or frequency of symptoms of infection or disease, elimination of symptoms and/or underlying cause, prevention of the occurrence of symptoms of infection and/or their underlying cause and improvement or remediation of damage. Collateral damage, for example, following viral infection may be liver damage such as cirrhosis or hepatocellular carcinoma of the liver. Conveniently, the treatment results in clearance of HBsAg via anti-HBsAg antibodies and resolution of HBV infection.

"Treating" a patient may involve prevention of an adverse physiological event in an individual as well as treatment of a clinically symptomatic individual by resolving HBV
infection or downstream condition such as liver damage or cancer. Thus, for example, the subject method of "treating" a patient with HBV infection treating chronic or persistent HBV infection and treating individuals presently on another therapeutic protocol.

Reference to "HBV" or its full term "Hepatitis B virus" includes all variants including variants resistant to particular therapeutic agents such as nucleoside or nucleotide analogs or immunological agents. Particularly important variants of HBV comprise mutations in HBsAg. Different strains of HBV are also contemplated by this term.

The term "patient" or "subject" or "individual" as used herein refers to an animal, preferably a mammal and more preferably a human who can benefit from the pharmaceutical formulations and methods of the present invention. There is no limitation on the type of animal that could benefit from the presently described pharmaceutical formulations and methods. A patient regardless of whether a human or non-human animal may be referred to as an individual, subject, animal, host or recipient. The compounds and methods of the present invention have applications in human medicine, veterinary medicine as well as in general, domestic or wild animal husbandry.

The compounds of the present invention which modulate the activity or levels of TLR& or a component in the TLR7 signaling pathway may be large or small molecules, nucleic acid molecules (including antisense or sense molecules), peptides, polypeptides or proteins or hybrid molecules such as RNAi- or siRNA-complexes (including RISC complexes and Dicer complexes), ribozymes or DNAzymes. The compounds may need to be modified so as to facilitate entry into a cell. Examples of agents include chemical agents which interact with or agonize TLR7 or genetic molecules which modulate TLR7 expression.

As indicated above, the preferred animals are humans.

Examples of laboratory test animals (including animal models) include mice, rats, rabbits, guinea pigs and hamsters. Rabbits and rodent animals, such as rats and mice, provide a convenient test system or animal model. Livestock animals include sheep, cows, pigs, goats, horses and donkeys. Non-mammalian animals such as avian species (such as ducks), zebrafish, amphibians (including cane toads) and Drosophila species such as Drosophila melanogaster are also contemplated.
The present invention provides, therefore, agents which agonize TLR7 levels or activity or a component in the TLR7 signaling pathway. The present invention contemplates methods of screening for such agents comprising, for example, contacting a candidate drug with TLR7 or a part thereof. The TLR7 molecule is referred to herein as a "target" or "target molecule". The screening procedure includes assaying (i) for the presence of a complex between the drug and the target, or (ii) an alteration in the expression levels of nucleic acid molecules encoding the target. One form of assay involves competitive binding assays. In such competitive binding assays, the target is typically labeled. Free target is separated from any putative complex and the amount of free (i.e. uncomplexed) label is a measure of the binding of the agent being tested to target molecule. One may also measure the amount of bound, rather than free, target. It is also possible to label the compound rather than the target and to measure the amount of compound binding to target in the presence and in the absence of the drug being tested.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to a target and is described in detail in Geysen (International Patent Publication No. WO 84/03564). Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with a target and washed. Bound target molecule is then detected by methods well known in the art. This method may be adapted for screening for non-peptide, chemical entities. This aspect, therefore, extends to combinatorial approaches to screening for target modulators of TLR7.

Purified target can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the target may also be used to immobilize the target on the solid phase. Antibodies specific for HBsAg may also be useful as inhibitors of HBsAg.

The present invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of specifically binding the target compete with a test compound for binding to the target or fragments thereof. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants of the target. Such antibodies may also be useful as diagnostic agents.

Antibodies to HBsAg or TLR7 or a component in the TLR7 signaling pathway may be
polyclonal or monoclonal although monoclonal antibodies are preferred. Antibodies may be prepared by any of a number of means. For the detection of HBsAg or TLR7 or a component in the TLR7 signaling pathway, antibodies are generally but not necessarily derived from non-human animals such as primates, livestock animals (e.g. sheep, cows, pigs, goats, horses), laboratory test animals (e.g. mice, rats, guinea pigs, rabbits) and companion animals (e.g. dogs, cats). Generally, antibody based assays are conducted in vitro on cell or tissue biopsies. However, if an antibody is suitably deimmunized or, in the case of human use, humanized, then the antibody can be labeled with, for example, a nuclear tag, administered to a subject and the site of nuclear label accumulation determined by radiological techniques.

The HBsAg or TLR7 or a component in the TLR7 signaling pathway antibody is regarded, therefore, as a pathogenic marker targeting agent. Accordingly, the present invention extends to deimmunized forms of the antibodies for use in pathogenic target imaging in human and non-human subjects. This is described further below.

For the generation of antibodies to HBsAg or TLR7 or a component in the TLR7 signaling pathway, the molecule is required to be extracted from a biological sample whether this is from an animal including human tissue or from cell culture if produced by recombinant means. Generally, monocytes and hepatocytes are a convenient source. The HBsAg or TLR7 or a component in the TLR7 signaling pathway can be separated from the biological sample by any suitable means. For example, the separation may take advantage of any one or more of the surface charge properties, size, density, biological activity and its affinity for another entity (e.g. another protein or chemical compound to which it binds or otherwise associates) with respect to HBsAg, TLR7 or a component in the TLR7 signaling pathway. Thus, for example, separation of HBsAg or TLR7 or a component in the TLR7 signaling pathway from the biological sample may be achieved by any one or more of ultra-centrifugation, ion-exchange chromatography (e.g. anion exchange chromatography, cation exchange chromatography), electrophoresis (e.g. polyacrylamide gel electrophoresis, isoelectric focussing), size separation (e.g., gel filtration, ultra-filtration) and affinity-mediated separation (e.g. immunoaffinity separation including, but not limited to, magnetic bead separation such as Dynabead (trademark) separation, immunochromatography, immuno-precipitation). Choice of the separation technique(s) employed may depend on the biological activity or physical properties of the HBsAg or TLR7 or a component in the TLR7 signaling pathway sought or from which tissues it is obtained.
Preferably, the separation of HBsAg or TLR7 or a component in the TLR7 signaling pathway from the biological fluid preserves conformational epitopes present on the kinase and, thus, suitably avoids techniques that cause denaturation of the molecule. Persons of skill in the art will recognize the importance of maintaining or mimicking as close as possible physiological conditions peculiar to HBsAg or TLR7 or a component in the TLR7 signaling pathway (e.g. the biological sample from which it is obtained) to ensure that the antigenic determinants or active site/s on the HBsAg or TLR7 or a component in the TLR7 signaling pathway, which are exposed to the animal, are structurally identical to that of the native molecule. This ensures the raising of appropriate antibodies in the immunized animal that would recognize the native molecule.

Immunization and subsequent production of monoclonal antibodies can be carried out using standard protocols as for example described in Kohler and Milstein, Nature. 256: 495-499, 1975; Kohler and Milstein, Eur. J. Immunol. 6(7): 511-519, 1976), Coligan et al. {"Current Protocols in Immunology, John Wiley & Sons, Inc., 1991-1997) and Toyama et al. {Monoclonal Antibody, Experiment Manual", published by Kodansha Scientific, 1987. Essentially, an animal is immunized with a pre-core protein/HBeAg or a TLR or a component in the TLR7 signaling pathway or a sample comprising HBsAg or TLR7 or a component in the TLR7 signaling pathway by standard methods to produce antibody-producing cells, particularly antibody-producing somatic cells (e.g. B lymphocytes). These cells can then be removed from the immunized animal for immortalization.

Where a fragment of HBsAg or TLR7 or a component in the TLR7 signaling pathway is used to generate antibodies, it may need to first be associated with a carrier. By "carrier" is meant any substance of typically high molecular weight to which a non- or poorly immunogenic substance (e.g. a hapten) is naturally or artificially linked to enhance its immunogenicity.

Immortalization of antibody-producing cells may be carried out using methods which are well-known in the art. For example, the immortalization may be achieved by the transformation method using Epstein-Barr virus (EBV) (Kozbor et al, Methods in Enzymology. 121: 140, 1986). In a preferred embodiment, antibody-producing cells are immortalized using the cell fusion method (described in Coligan et al, supra, 1991-1997), which is widely employed for the production of monoclonal antibodies. In this method, somatic antibody-producing cells with the potential to produce antibodies, particularly B cells, are fused with a myeloma cell line. These somatic cells may be derived from the lymph
nodes, spleens and peripheral blood of primed animals, preferably rodent animals such as mice and rats. Mice spleen cells are particularly useful. It would be possible, however, to use rat, rabbit, sheep or goat cells, or cells from other animal species instead.

Specialized myeloma cell lines have been developed from lymphocytic tumors for use in hybridoma-producing fusion procedures (Kohler and Milstein, *supra* 1976; Shulman *et al.*, *Nature*. 276:269-270, 1978; Volk *et al.*, *J. Virol.* 42(1):220-227, 1982). These cell lines have been developed for at least three reasons. The first is to facilitate the selection of fused hybridomas from unfused and similarly indefinitely self-propagating myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocytic tumor cells to produce their own antibodies. To eliminate the production of tumor cell antibodies by the hybridomas, myeloma cell lines incapable of producing endogenous light or heavy immunoglobulin chains are used. A third reason for selection of these cell lines is for their suitability and efficiency for fusion.

Many myeloma cell lines may be used for the production of fused cell hybrids, including, e.g. P3X63-Ag8, P3X63-AG8.653, P3/NS1-Ag4-1 (NS-I), Sp2/0-Agl4 and S194/5.XXO.Bu.l. The P3X63-Ag8 and NS-I cell lines have been described by Kohler and Milstein (*supra*, 1976). Shulman *et al.*, (*supra*, 1978) developed the Sp2/0-Agl4 myeloma line. The S194/5.XXO.Bu.l line was reported by Trowbridge (*J. Exp. Med.* 148(1): 313-323, 1978).

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually involve mixing somatic cells with myeloma cells in a 10:1 proportion (although the proportion may vary from about 20:1 to about 1:1), respectively, in the presence of an agent or agents (chemical, viral or electrical) that promotes the fusion of cell membranes. Fusion methods have been described (Kohler and Milstein, *supra*, 1975.; Kohler and Milstein, *supra*, 1976; Gefter *et al.*, *Somatic Cell Genet.* 3: 231-236, 1977; Volk *et al.*, *supra*, 1982). The fusion-promoting agents used by those investigators were Sendai virus and polyethylene glycol (PEG).

Because fusion procedures produce viable hybrids at very low frequency (e.g. when spleens are used as a source of somatic cells, only one hybrid is obtained for roughly every $1 \times 10^5$ spleen cells), it is preferable to have a means of selecting the fused cell hybrids from the remaining unfused cells, particularly the unfused myeloma cells. A means of detecting the
desired antibody-producing hybridomas among other resulting fused cell hybrids is also necessary. Generally, the selection of fused cell hybrids is accomplished by culturing the cells in media that support the growth of hybridomas but prevent the growth of the unfused myeloma cells, which normally would go on dividing indefinitely. The somatic cells used in the fusion do not maintain long-term viability in in vitro culture and hence do not pose a problem. In the example of the present invention, myeloma cells lacking hypoxanthine phosphoribosyl transferase (HPRT-negative) were used. Selection against these cells is made in hypoxanthine/aminopterin/thymidine (HAT) medium, a medium in which the fused cell hybrids survive due to the HPRT-positive genotype of the spleen cells. The use of myeloma cells with different genetic deficiencies (drug sensitivities, etc.) that can be selected against in media supporting the growth of genotypically competent hybrids is also possible.

Several weeks are required to selectively culture the fused cell hybrids. Early in this time period, it is necessary to identify those hybrids which produce the desired antibody, so that they may subsequently be cloned and propagated. Generally, around 10% of the hybrids obtained produce the desired antibody, although a range of from about 1 to about 30% is not uncommon. The detection of antibody-producing hybrids can be achieved by any one of several standard assay methods, including enzyme-linked immunoassay and radioimmunoassay techniques as, for example, described in Kennet et al. (Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses, pp 376-384, Plenum Press, New York, 1980) and by FACS analysis (O'Reilly et al., Biotechniques. 25: 824-830, 1998).

Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing cell lines, each cell line may be propagated in either of two standard ways. A suspension of the hybridoma cells can be injected into a histocompatible animal. The injected animal will then develop tumors that secrete the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propagated in vitro in laboratory culture vessels. The culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration or centrifugation, and subsequently purified.

The cell lines are tested for their specificity to detect HBsAg or TLR7 or a component in the TLR7 signaling pathway of interest by any suitable immunodetection means. For example,
cell lines can be aliquoted into a number of wells and incubated and the supernatant from each well is analyzed by enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody technique, or the like. The cell line(s) producing a monoclonal antibody capable of recognizing the target HBsAg or TLR7 or a component in the TLR7 signaling pathway but which does not recognize non-target epitopes are identified and then directly cultured in vitro or injected into a histocompatible animal to form tumors and to produce, collect and purify the required antibodies.

These antibodies are HBsAg- or TLR7-specific or specific to a component of the TLR7 signaling pathway. This means that the antibodies are capable of distinguishing a HBsAg or TLR7 or a component in the TLR7 signaling pathway from other molecules. Additional broad spectrum antibodies may be used provided that they do not cross-react with molecules in a normal cell.

Where the monoclonal antibody is destined for use as a therapeutic agent such as to activate TLR7 or a component in the TLR7 signaling pathway or inhibit HBsAg, then, it will need to be deimmunized with respect to the host into which it will be introduced (e.g. a human). The deimmunization process may take any of a number of forms including the preparation of chimeric antibodies which have the same or similar specificity as the monoclonal antibodies prepared according to the present invention. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin variable and constant region genes belonging to different species. Thus, in accordance with the present invention, once a hybridoma producing the desired monoclonal antibody is obtained, techniques are used to produce interspecific monoclonal antibodies wherein the binding region of one species is combined with a non-binding region of the antibody of another species (Liu et al, Proc. Natl. Acad. Sci. USA. 84: 3439-3443, 1987). For example, complementary determining regions (CDRs) from a non-human (e.g. murine) monoclonal antibody can be grafted onto a human antibody, thereby "humanizing" the murine antibody (European Patent No. 0 239 400; Jones et al, Nature. 321: 522-525, 1986; Verhoeyen et al, Science. 239: 1534-1536, 1988; Richmann et al, Nature. 332: 323-327, 1988). In this case, the deimmunizing process is specific for humans. More particularly, the CDRs can be grafted onto a human antibody variable region with or without human constant regions. The non-human antibody providing the CDRs is typically referred to as the "donor" and the human antibody providing the framework is typically referred to as the "acceptor". Constant regions need not be present, but if they are, they must be substantially identical to
human immunoglobulin constant regions, i.e. at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a humanized antibody, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. Thus, a "humanized antibody" is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. A donor antibody is said to be "humanized", by the process of "humanization", because the resultant humanized antibody is expected to bind to the same antigen as the donor antibody that provides the CDRs. Reference herein to "humanized" includes reference to an antibody deimmunized to a particular host, in this case, a human host.

It will be understood that the deimmunized antibodies may have additional conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. Exemplary conservative substitutions may be made according to Table 2.
TABLE 2

<table>
<thead>
<tr>
<th>ORIGINAL RESIDUE</th>
<th>EXEMPLARY SUBSTITUTIONS</th>
</tr>
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<tbody>
<tr>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>Arg</td>
<td>Lys</td>
</tr>
<tr>
<td>Asn</td>
<td>Gln, His</td>
</tr>
<tr>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>Cys</td>
<td>Ser</td>
</tr>
<tr>
<td>Gln</td>
<td>Asn</td>
</tr>
<tr>
<td>Glu</td>
<td>Asp</td>
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<td>Asn, Gln</td>
</tr>
<tr>
<td>Ile</td>
<td>Leu, Val</td>
</tr>
<tr>
<td>Leu</td>
<td>Ile, Val</td>
</tr>
<tr>
<td>Lys</td>
<td>Arg, Gln, Glu</td>
</tr>
<tr>
<td>Met</td>
<td>Leu, Ile</td>
</tr>
<tr>
<td>Phe</td>
<td>Met, Leu, Tyr</td>
</tr>
<tr>
<td>Ser</td>
<td>Thr</td>
</tr>
<tr>
<td>Thr</td>
<td>Ser</td>
</tr>
<tr>
<td>Trp</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyr</td>
<td>Trp, Phe</td>
</tr>
<tr>
<td>Val</td>
<td>Ile, Leu</td>
</tr>
</tbody>
</table>

Exemplary methods which may be employed to produce deimmunized antibodies according to the present invention are described, for example, in Richmann et al, supra, 1988; European Patent No. 0 239 400; U.S. Patent No. 6,056,957, U.S. Patent No. 6,180,370, U.S. Patent No. 6,180,377.

Thus, in one embodiment, the present invention contemplates a deimmunized antibody molecule having specificity for an epitope recognized by a monoclonal antibody to TLR7 or a component in the TLR7 signaling pathway or in HBsAg wherein at least one of the CDRs of the variable domain of said deimmunized antibody is derived from the said monoclonal antibody to the TLR7 or a component in the TLR7 signaling pathway or HBsAg and the remaining immunoglobulin-derived parts of the deimmunized antibody molecule are derived...
from an immunoglobulin or an analog thereof from the host for which the antibody is to be deimmunized.

This aspect of the present invention involves manipulation of the framework region of a non-human antibody.

The present invention extends to mutants and derivatives of the subject antibodies but which still retain specificity for TLR7 or a component in the TLR7 signaling pathway or HBsAg.

The terms "mutant" or "derivatives" includes one or more amino acid substitutions, additions and/or deletions.

As used herein, the term "CDR" includes CDR structural loops which covers to the three light chain and the three heavy chain regions in the variable portion of an antibody framework region which bridge β strands on the binding portion of the molecule. These loops have characteristic canonical structures (Chothia et al, J. Mol. Biol. 196: 901, 1987; Chothia et al, J. Mol. Biol. 227: 799, 1992).

By "framework region" is meant region of an immunoglobulin light or heavy chain variable region, which is interrupted by three hypervariable regions, also called CDRs. The extent of the framework region and CDRs have been precisely defined (see, for example, Kabat et al, "Sequences of Proteins of Immunological Interest", U.S. Department of Health and Human Sciences, 1983). The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. As used herein, a "human framework region" is a framework region that is substantially identical (about 85% or more, usually 90-95% or more) to the framework region of a naturally occurring human immunoglobulin. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs. The CDRs are primarily responsible for binding to an epitope of TLR7 or a component in the TLR7 signaling pathway or HBsAg.

As used herein, the term "heavy chain variable region" means a polypeptide which is from about 110 to 125 amino acid residues in length, the amino acid sequence of which corresponds to that of a heavy chain of a monoclonal antibody of the invention, starting from the amino-terminal (N-terminal) amino acid residue of the heavy chain. Likewise, the term
"light chain variable region" means a polypeptide which is from about 95 to 130 amino acid residues in length, the amino acid sequence of which corresponds to that of a light chain of a monoclonal antibody of the invention, starting from the N-terminal amino acid residue of the light chain. Full-length immunoglobulin "light chains" (about 25 Kd or 214 amino acids) are encoded by a variable region gene at the NH₂-terminus (about 110 amino acids) and a K or λ constant region gene at the COOH-terminus. Full-length immunoglobulin "heavy chains" (about 50 Kd or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, e.g. γ (encoding about 330 amino acids).

The term "immunoglobulin" or "antibody" is used herein to refer to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the κ, λ, α, γ (IgGi, IgG₂, IgG₃, IgG₄), δ, ε and μ constant region genes, as well as the myriad immunoglobulin variable region genes. One form of immunoglobulin constitutes the basic structural unit of an antibody. This form is a tetramer and consists of two identical pairs of immunoglobulin chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the antibody effector functions. In addition to antibodies, immunoglobulins may exist in a variety of other forms including, for example, Fv, Fab, Fab' and (Fab')₂.

The present invention also contemplates the use and generation of fragments of monoclonal antibodies produced by the method of the present invention including, for example, Fv, Fab, Fab' and F(ab')₂ fragments. Such fragments may be prepared by standard methods as for example described by Coligan et al. (supra, 1991-1997).

The present invention also contemplates synthetic or recombinant antigen-binding molecules with the same or similar specificity as the monoclonal antibodies of the invention. Antigen-binding molecules of this type may comprise a synthetic stabilized Fv fragment. Exemplary fragments of this type include single chain Fv fragments (sFv, frequently termed scFv) in which a peptide linker is used to bridge the N terminus or C terminus of a V₇ domain with the C terminus or N-terminus, respectively, of a Vλ domain. ScFv lack all constant parts of whole antibodies and are not able to activate complement. Suitable peptide linkers for joining the Y₇ and Vλ domains are those which allow the V₇ and Vλ domains to fold into a single
polypeptide chain having an antigen binding site with a three dimensional structure similar to that of the antigen binding site of a whole antibody from which the Fv fragment is derived. Linkers having the desired properties may be obtained by the method disclosed in U.S. Patent No 4,946,778. However, in some cases a linker is absent. ScFvs may be prepared, for example, in accordance with methods outlined in Krebber et al. J. Immunol. Methods. 201(1):35-55, 1997. Alternatively, they may be prepared by methods described in U.S. Patent No 5,091,513, European Patent No 239,400 or the articles by Winter and Milstein (Nature. 349:293, 1991) and Plückthun et al. (In Antibody engineering: A practical approach, 203-252, 1996).

Alternatively, the synthetic stabilized Fv fragment comprises a disulphide stabilized Fv (dsFv) in which cysteine residues are introduced into the VH and VL domains such that in the fully folded Fv molecule the two residues will form a disulphide bond there between. Suitable methods of producing dsFv are described, for example, in (Glockshuber et al, Biochem. 29:1363-1367, 1990; Reiter et al, J. Biol. Chem. 269;15327-18331, 1994; Reiter et al, Biochem. 33:5451-5459, 1994; Reiter et al, Cancer Res. 54:2714-2718, 1994; Webber et al, Mol. Immunol. 32:249-258, 1995).

Also contemplated as synthetic or recombinant antigen-binding molecules are single variable region domains (termed dAbs) as, for example, disclosed in (Ward et al, Nature. 341:544-546, 1989; Hamers-Casterman et al, Nature. 363:446-448, 1993; Davies and Riechmann, FEBS Lett. 339:285-290, 1994).

Alternatively, the synthetic or recombinant antigen-binding molecule may comprise a "minibody". In this regard, minibodies are small versions of whole antibodies, which encode in a single chain the essential elements of a whole antibody. Suitably, the minibody is comprised of the VH and VL domains of a native antibody fused to the hinge region and CH3 domain of the immunoglobulin molecule as, for example, disclosed in U.S. Patent No 5,837,821.

In an alternate embodiment, the synthetic or recombinant antigen binding molecule may comprise non-immunoglobulin derived, protein frameworks. For example, reference may be made to (Ku & Schutz, Proc. Natl. Acad. Sci. USA. 92:6552-6556, 1995) which discloses a four-helix bundle protein cytochrome b562 having two loops randomized to create CDRs, which have been selected for antigen binding.
The synthetic or recombinant antigen-binding molecule may be multivalent (i.e. having more
than one antigen binding site). Such multivalent molecules may be specific for one or more
antigens. Multivalent molecules of this type may be prepared by dimerization of two antibody
fragments through a cysteinylation-containing peptide as, for example disclosed by (Adams et al.
Cancer Res. 53:4026-4034, 1993; Cumber et al, J. Immunol. 149:120-126, 1992). Alternatively, dimerization may be facilitated by fusion of the antibody fragments to
amphphilic helices that naturally dimerize (Plünckthun, Biochem 37:1579-1584, 1992) or by
use of domains (such as leucine zippers jun and fos) that preferentially heterodimerize
(Kostelny et al, J. Immunol. 148:1547-1553, 1992). Multivalent antibodies are useful, for
example, in detecting different forms of TLRs such as TLR-2 and TLR-4.

Analogs of proteinaceous molecules (e.g. ligands of TLR7 or a component in the TLR7
signaling pathway) contemplated herein include but are not limited to modification to side
chains, incorporating of unnatural amino acids and/or their derivatives during peptide,
polypeptide or protein synthesis and the use of crosslinkers and other methods which impose
conformational constraints on the proteinaceous molecule or their analogs.

Examples of side chain modifications contemplated by the present invention include
modifications of amino groups such as by reductive alkylation by reaction with an aldehyde
followed by reduction with NaBH₄; amidation with methylacetimide; acylation with
acetic anhydride; carboxymylation of amino groups with cyanate; trinitrobenzylation of amino
groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with
succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with
pyridoxal-5-phosphate followed by reduction with NaBH₄.

The guanidine group of arginine residues may be modified by the formation of heterocyclic
condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carboximide activation via O-acylisourea formation
followed by subsequent derivitization, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic
acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed
disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other
substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

5 Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

10 Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sарcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table 3.

20

<table>
<thead>
<tr>
<th>Non-conventional amino acid</th>
<th>Code</th>
<th>Non-conventional amino acid</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-aminobutyric acid</td>
<td>Abu</td>
<td>L-N-methylalanine</td>
<td>Nmala</td>
</tr>
<tr>
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<td>Mgabu</td>
<td>L-N-methylarginine</td>
<td>Nmarg</td>
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<tr>
<td>aminocyclopropane-carboxylate</td>
<td>Cpro</td>
<td>L-N-methyasparagine</td>
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<td>Norb</td>
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<td>Nmgln</td>
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<td>Chexa</td>
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Crosslinkers can be used, for example, to stabilize 3D conformations, using homobifunctional crosslinkers such as the bifunctional imido esters having (CHa)n spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of Cα and Nα-methylamino acids, introduction of double bonds between Cα and Cβ atoms of amino acids and the formation of cyclic peptides or analogs by introducing covalent bonds such as forming an amide bond between the N and C termini,
between two side chains or between a side chain and the N or C terminus.

Accordingly, another aspect of the present invention contemplates any compound which binds or otherwise interacts with TLR7 or a component of the TLR7 signaling pathway resulting in enhanced clearance of HBsAg and resolution of HBV infection.

Another useful group of compounds is a mimetic. The terms "peptide mimetic", "target mimetic" or "mimetic" are intended to refer to a substance which has some chemical similarity to the target but which antagonizes or agonizes or mimics the target. The target in this case may be a ligand of TLR7 or a component in the TLR7 signaling pathway or HBsAg. A peptide mimetic may be a peptide-containing molecule that mimics elements of protein secondary structure (Johnson et al, "Peptide Turn Mimetics" in Biotechnology and Pharmacy, Pezzuto et al. Eds., Chapman and Hall, New York, 1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions such as those of antibody and antigen, enzyme and substrate or scaffolding proteins. A peptide mimetic is designed to permit molecular interactions similar to the natural molecule. Peptide or non-peptide mimetics may be useful, for example, to competitively inhibit or otherwise bind to TLR7 or a component in the TLR7 signaling pathway or HBsAg.

Again, the compounds of the present invention may be selected to interact with a target alone or single or multiple compounds may be used to affect multiple targets. For example, multiple targets may include an HBsAg and HBV. For example, one useful therapeutic combination would be an agonist of TLR7 or a component in the TLR7 signaling pathway and a nucleoside or nucleotide analog and/or antiviral cytokine

The target or fragment employed in screening assays may either be free in solution, affixed to a solid support, or borne on a cell surface. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant polynucleotides expressing TLR7 or a component in the TLR7 signaling pathway or a fragment thereof, preferably in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays.

A substance identified as a modulator of target function or gene activity may be a peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many in vivo
pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

The designing of mimetics to a pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesize or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to he quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large numbers of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. First, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptides are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modeled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, x-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modeling process.

In a variant of this approach, the three-dimensional structure of TLR7 or a component in the TLR7 signaling pathway and a ligand thereof are modeled. This can be especially useful where the TLR7 or a component in the TLR7 signaling pathway and/or its ligand change conformation on binding, allowing the model to take account of this in the design of the mimetic. Modeling can be used to generate inhibitors which interact with the linear sequence or a three-dimensional configuration.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted onto
it can conveniently be selected so that the mimetic is easy to synthesize, is likely to be pharmacologically acceptable, and does not degrade in vivo, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is peptide-based, further stability can be achieved by cyclizing the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimization or modification can then be carried out to arrive at one or more final mimetics for in vivo or clinical testing.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact (e.g. agonists, antagonists, inhibitors or enhancers) in order to fashion drugs which are, for example, more active or stable forms of the polypeptide, or which, e.g. enhance or interfere with the function of a polypeptide in vivo. See, e.g. Hodgson (Bio/Technology. P:19-21, 1991). In one approach, one first determines the three-dimensional structure of TLR7, TLR7 ligand or HBsAg by x-ray crystallography, by computer modeling or most typically, by a combination of approaches. Useful information regarding the structure of TLR7, TLR7 ligand or HBsAg may also be gained by modeling based on the structure of homologous proteins. An example of rational drug design is the development of HIV protease inhibitors (Erickson et al, Science. 249:527-533, 1990). In addition, target molecules may be analyzed by an alanine scan (Wells, Methods Enzymol. 202:2699-2705, 1991). In this technique, an amino acid residue is replaced by Ala and its effect on the peptide's activity is determined. Each of the amino acid residues of the peptide is analyzed in this manner to determine the important regions of the peptide.

It is also possible to isolate an antibody to TLR7 or a component in the TLR7 signaling pathway (such as by the method described above) and then to solve its crystal structure. In principle, this approach yields a pharmacophore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analog of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically or biologically produced banks of peptides. Selected peptides would then act as the pharmacophore.

The present invention extends to a genetic approach to up-regulating expression of a gene
encoding a TLR7 or a component in the TLR7 signaling pathway. General techniques can be used to up-regulate expression of TLR7 or a component in the TLR7 signaling pathway by increasing gene copy numbers or antagonizing inhibitors of gene expression.

The terms "nucleic acids", "nucleotide" and "polynucleotide" include RNA, cDNA, genomic DNA, synthetic forms and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog (such as the morpholine ring), internucleotide modifications such as uncharged linkages (e.g. methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g. phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g. polypeptides), intercalators (e.g. acridine, psoralen, etc.), chelators, alkylators and modified linkages (e.g. α-anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen binding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

Antisense polynucleotide sequences, for example, are useful in silencing transcripts of the HBsAg gene. Expression of such an antisense construct within a cell interferes with HBsAg gene transcription and/or translation. Furthermore, co-suppression and mechanisms to induce RNAi such as using short interfering RNA (siRNA) or ONA-derived RNAi (ddRNAi) may also be employed. Hence, antisense or sense molecules may be directly administered. In this latter embodiment, the antisense or sense molecules may also be formulated in a composition and then administered by any number of means to target cells or administered via an expression construct.

A variation on antisense and sense molecules involves the use of morpholinos, which are oligonucleotides composed of morpholine nucleotide derivatives and phosphorodiamidate linkages (for example, Summerton and Weller, *Antisense and Nucleic Acid Drug Development*. 7:187-195, 1997). Such compounds are injected into embryos and the effect of interference with mRNA is observed.
In one embodiment, the present invention employs compounds such as oligonucleotides and similar species for use in modulating the function or effect of nucleic acid molecules such as those encoding a HBsAg, i.e. the oligonucleotides induce pre-transcriptional or post-transcriptional gene silencing. This is accomplished by providing oligonucleotides which specifically hybridize to, or have complementing with a nucleic acid molecule encoding the HBsAg. The oligonucleotides may be provided directly to a cell or generated within the cell. As used herein, the terms "target nucleic acid" and "nucleic acid molecule encoding the HBsAg gene transcript" have been used for convenience to encompass DNA encoding the HBsAg, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. The hybridization of a compound of the subject invention with its target nucleic acid is generally referred to as "antisense" or may be part of a complex with dicer such as a RISC.

In the context of this invention, "hybridization" means the pairing of complementary strands of oligomeric compounds. In the present invention, the preferred mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases) of the strands of oligomeric compounds. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. Hybridization can occur under varying circumstances.

An antisense or RNAi compound is specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target nucleic acid sequences under conditions in which specific binding is desired, i.e. under physiological conditions in the case of in vivo assays or therapeutic treatment, and under conditions in which assays are performed in the case of in vitro assays.

"Complementary" as used herein, refers to the capacity for precise pairing between two nucleobases of an oligomeric compound. For example, if a nucleobase at a certain position of an oligonucleotide (an oligomeric compound), is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, said target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position.

The oligonucleotide and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

In the context of the subject invention, the term "oligomeric compound" refers to a polymer or oligomer comprising a plurality of monomeric units. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics, chimeras, analogs and homologs thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for a target nucleic acid and increased stability in the presence of nucleases.

While oligonucleotides are a preferred form of the compounds of this invention, the present invention comprehends other families of compounds as well, including but not limited to oligonucleotide analogs and mimetics such as those herein described.

The open reading frame (ORF) or "coding region" which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is a region which may be effectively targeted. Within the context of the present invention, one region is the intragenic region encompassing the translation initiation or termination codon of the open reading frame (ORF) of a gene.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the
phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may, therefore, fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense or RNAi compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphorotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

The antisense or RNAi oligonucleotides may be administered by any convenient means including by inhalation, local or systemic means.

In an alternative embodiment, genetic constructs including DNA vaccines are used to generate antisense or ddRNAi molecules in vivo.
Following identification of an agent which interacts with TLR7 or component of the TLR7 signaling pathway, it may be manufactured and/or used in a preparation, i.e. in the manufacture or formulation or a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals in a method of treatment of infection. Alternatively, they may be incorporated into a patch or slow release capsule or implant.

Thus, the present invention extends, therefore, to a pharmaceutical composition, medicament, drug or other composition including a patch or slow release formulation comprising a modulator of TLR7 activity or gene expression or the activity or gene expression of a component of the TLR7 signaling pathway.

Another aspect of the present invention contemplates a method comprising administration of such a composition to a subject such as for treatment or prophylaxis of an infection or other disease condition. Furthermore, the present invention contemplates a method of making a pharmaceutical composition comprising admixing an agent of the instant invention with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients. Where multiple compositions are provided, then such compositions may be given simultaneously or sequentially. Sequential administration includes administration within nanoseconds, seconds, minutes, hours or days. Preferably, sequential administration is within seconds or minutes.

Multi-part including two-art pharmaceutical compositions or packs are also contemplated comprising multiple components such as those which agonize TLR7 or a component in the TLR7 signaling pathway and another anti-HBV agent. Anti-HBV agents include nucleoside or nucleotide analogs, anti-HBsAg antibodies and cytokines as well as an antigen capable of stimulating an immune response to HBV and/or its components. Such multi-part pharmaceutical compositions or packs may maintain different agents or groups of agents separately. These are either dispensed separately or admixed prior to being dispensed.

Accordingly, another aspect of the present invention contemplates a method for the treatment or prophylaxis of an infection or other disease condition in a subject, said method comprising administering to said subject an effective amount of a compound as described herein or a composition comprising same.
Preferably, the subject is a mammal such as a human or an animal model system such as a mouse, rat, rabbit, guinea pig, hamster, zebrafish or amphibian or avian species such as a duck.

This method also includes providing a wild-type or mutant target gene function to a cell. This is particularly useful when generating an animal model. Alternatively, it may be part of a gene therapy approach. A target gene or a part of the gene may be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation, the gene will be expressed by the cell from the extrachromosomal location. If a gene portion is introduced and expressed in a cell carrying a mutant target allele, the gene portion should encode a part of the target protein. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art and any suitable vector may be used. Methods for introducing DNA into cells such as electroporation calcium phosphate co-precipitation and viral transduction are known in the art. This aspect of the present invention extends to constructs which encode ddRNAi.

Non-viral gene transfer methods are known in the art such as chemical techniques including calcium phosphate co-precipitation, mechanical techniques, for example, microinjection, membrane fusion-mediated transfer via liposomes and direct DNA uptake and receptor-mediated DNA transfer. Viral-mediated gene transfer can be combined with direct in vivo gene transfer using liposome delivery, allowing one to direct the viral vectors to particular cells. Alternatively, the retroviral vector producer cell line can be injected into particular tissue. Injection of producer cells would then provide a continuous source of vector particles.

In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization and degradation of the endosome before the coupled DNA is damaged. For other techniques for the delivery of adenovirus based vectors, see U.S. Patent No. 5,691,198.

Liposome/DNA complexes have been shown to be capable of mediating direct in vivo gene transfer. While in standard liposome preparations the gene transfer process is non-specific, localized in vivo uptake and expression have been reported in tumor deposits, for example, following direct in situ administration.

If the polynucleotide encodes a sense or antisense polynucleotide or a ribozyme or DNAzyme, expression will produce the sense or antisense polynucleotide or ribozyme or DNAzyme. Thus, in this context, expression does not require that a protein product be synthesized. In addition to the polynucleotide cloned into the expression vector, the vector also contains a promoter functional in eukaryotic cells. The cloned polynucleotide sequence is under control of this promoter. Suitable eukaryotic promoters include those described above. The expression vector may also include sequences, such as selectable markers and other sequences described herein.
Cells which carry a mutant target gene can be used as model systems to study the effects of infection or other disease condition.

The compounds, agents, medicaments, nucleic acid molecules and other target antagonists or agonists of the present invention can be formulated in pharmaceutical compositions which are prepared according to conventional pharmaceutical compounding techniques. See, for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Company, Easton, PA, U.S.A.). The composition may contain the active agent or pharmaceutically acceptable salts of the active agent. These compositions may comprise, in addition to one of the active substances, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g. topical, intravenous, oral, intrathecal, epineural or parenteral.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active agent can be encapsulated to make it stable to passage through the gastrointestinal tract while at the same time allowing for passage across the blood brain barrier. See for example, International Patent Publication No. WO 96/11698.

For parenteral administration, the compound may be dissolved in a pharmaceutical carrier and administered as either a solution of a suspension. Illustrative of suitable carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives,
suspending agents, solubilizing agents, buffers and the like. When the compounds are being administered intrathecally, they may also be dissolved in cerebrospinal fluid.

The active agent is preferably administered in a therapeutically effective amount. The actual amount administered and the rate and time-course of administration will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc. is within the responsibility of general practitioners or specialists and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in Remington's Pharmaceutical Sciences, supra.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibodies or cell specific ligands or specific nucleic acid molecules. Targeting may be desirable for a variety of reasons, e.g. if the agent is unacceptably toxic or if it would otherwise require too high a dosage or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they could be produced in the target cell, e.g. in a viral vector such as described above or in a cell based delivery system such as described in U.S. Patent No. 5,550,050 and International Patent Publication Nos. WO 92/19195, WO 94/25503, WO 95/01203, WO 95/05452, WO 96/02286, WO 96/02646, WO 96/40871, WO 96/40959 and WO 97/12635. The vector could be targeted to the target cells. The cell based delivery system is designed to be implanted in a patient's body at the desired target site and contains a coding sequence for the target agent. Alternatively, the agent could be administered in a precursor form for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. See, for example, European Patent Application No. 0 425 731A and International Patent Publication No. WO 90/07936.

The present invention further contemplates diagnostic protocols such as to determine the presence or absence of infection or other disease condition, whether an infection has become chronic, the susceptibility of a subject to infection and/or the efficacy of a therapeutic protocol.

Immunological based TLR7 or a component in the TLR7 signaling pathway or HBsAg
detection protocols may take a variety of forms. For example, a plurality of antibodies may be immobilized in an array each with different specificities to particular TLR7 or a component in the TLR7 signaling pathway or HBsAg or monocytes or hepatocytes comprising TLR7 or a component in the TLR7 signaling pathway or HBsAg. Cells from a biopsy are then brought into contact with the antibody array and a diagnosis may be made as to the level and type of TLR7 or a component in the TLR7 signaling pathway or HBsAg elevated or down-regulated on or in the cell.

Other more conventional assays may also be conducted such as by ELISA, Western blot analysis, immunoprecipitation analysis, immunofluorescence analysis, immunochemistry analysis or FACS analysis.

The present invention provides, therefore, a method of detecting in TLR7 or a component in the TLR7 signaling pathway or HBsAg or cell comprising same or fragment, variant or derivative thereof comprising contacting the sample with an antibody or fragment or derivative thereof and detecting the level of a complex comprising said antibody and the TLR7 or a component in the TLR7 signaling pathway or HBsAg or fragment, variant or derivative thereof compared to normal controls wherein altered levels of the TLR7 or a component in the TLR7 signaling pathway or HBsAg is indicative of the presence or absence of infection or other disease condition.

As discussed above, any suitable technique for determining formation of the complex may be used. For example, an antibody according to the invention, having a reporter molecule associated therewith, may be utilized in immunoassays. Such immunoassays include but are not limited to radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic techniques (ICTs), Western blotting which are well known to those of skill in the art. For example, reference may be made to Coligan et al, supra, 1991-1997, which discloses a variety of immunoassays which may be used in accordance with the present invention. Immunoassays may include competitive assays. It will be understood that the present invention encompasses qualitative and quantitative immunoassays.

Suitable immunoassay techniques are described, for example, in U.S. Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These include both single-site and two-site assays of the non-competitive types, as well as the traditional competitive binding assays. These assays also include direct binding of a labeled antigen-binding molecule to a target antigen. The antigen
in this case is the TLR or a fragment thereof or a component in the TLR7 signaling pathway.

Two-site assays are particularly favoured for use in the present invention. A number of variations of these assays exist, all of which are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabeled antigen-binding molecule such as an unlabeled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, another antigen-binding molecule, suitably a second antibody specific to the antigen, labeled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labeled antibody. Any unreacted material is washed away and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may be either qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include a simultaneous assay, in which both sample and labeled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including minor variations as will be readily apparent.

In the typical forward assay, a first antibody having specificity for the antigen or antigenic parts thereof is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient and under suitable conditions to allow binding of any antigen present to the antibody. Following the incubation period, the antigen-antibody complex is washed and dried and incubated With a second antibody specific for a portion of the antigen. The second antibody has generally a reporter molecule associated therewith that is used to indicate the binding of the second antibody to the antigen. The amount of labeled antibody that binds, as determined by the associated reporter molecule, is proportional to the amount of antigen bound to the immobilized first antibody.
An alternative method involves immobilizing the antigen in the biological sample and then exposing the immobilized antigen to specific antibody that may or may not be labeled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound antigen may be detectable by direct labelling with the antibody. Alternatively, a second labeled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

From the foregoing, it will be appreciated that the reporter molecule associated with the antigen-binding molecule may include the following:-

(a) direct attachment of the reporter molecule to the antibody;

(b) indirect attachment of the reporter molecule to the antibody; i.e., attachment of the reporter molecule to another assay reagent which subsequently binds to the antibody; and

(c) attachment to a subsequent reaction product of the antibody.

The reporter molecule may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorochrome, a chemiluminescent molecule, a paramagnetic ion, a lanthanide ion such as Europium (Eu³⁺), a radioisotope including other nuclear tags and a direct visual label.

In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

A large number of enzymes suitable for use as reporter molecules is disclosed in U.S. Patent Nos. 4,366,241, 4,843,000, and 4,849,338. Suitable enzymes useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzymes may be used alone or in combination with a second enzyme that is in solution.
Suitable fluorochromes include, but are not limited to, fluorescein isothiocyanate (FITC),
tetramethylrhodamine isothiocyanate (TRITC), R-Phycoerythrin (RPE), and Texas Red.
Other exemplary fluorochromes include those discussed by International Patent Publication
No. WO 93/06121. Reference also may be made to the fluorochromes described in U.S.
Patent Nos. 5,573,099 and 5,326,692. Alternatively, reference may be made to the
fluorochromes described in U.S. Patent Nos. 5,227,487, 5,274,113, 5,405,975, 5,433,896,

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody,
generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a
wide variety of different conjugation techniques exist which are readily available to the
skilled artisan. The substrates to be used with the specific enzymes are generally chosen for
the production of, upon hydrolysis by the corresponding enzyme, a detectable colour change.
Examples of suitable enzymes include those described supra. It is also possible to employ
fluorogenic substrates, which yield a fluorescent product rather than the chromogenic
substrates noted above. In all cases, the enzyme-labeled antibody is added to the first
antibody-antigen complex, allowed to bind, and then the excess reagent washed away. A
solution containing the appropriate substrate is then added to the complex of antibody-
antigen-antibody. The substrate will react with the enzyme linked to the second antibody,
giving a qualitative visual signal, which may be further quantitated, usually
spectrophotometrically, to give an indication of the amount of antigen which was present in
the sample.

Alternately, fluorescent compounds, such as fluorescein, rhodamine and the lanthanide,
europium (EU), may be chemically coupled to antibodies without altering their binding
capacity. When activated by illumination with light of a particular wavelength, the
fluorochrome-labeled antibody adsorbs the light energy, inducing a state to excitability in the
molecule, followed by emission of the light at a characteristic colour visually detectable with
a light microscope. The fluorescent-labeled antibody is allowed to bind to the first antibody-
antigen complex. After washing off the unbound reagent, the remaining tertiary complex is
then exposed to light of an appropriate wavelength. The fluorescence observed indicates the
presence of the antigen of interest. Immunofluorometric assays (IFMA) are well established
in the art and are particularly useful for the present method. However, other reporter
molecules, such as radioisotope, chemiluminescent or bioluminescent molecules may also be
employed.
Monoclonal antibodies to TLR7 or a component in the TLR7 signaling pathway or HBsAg may also be used in ELISA-mediated detection of the TLR. This may be undertaken in any number of ways such as immobilizing anti-TLR7 or antibodies to a component of the TLR7 signaling pathway or anti-HBsAg antibodies to a solid support and contacting these with liver cells. Labeled anti-TLR7 or anti-component of TLR7 signaling pathway or anti-HBsAg antibodies are then used to detect immobilized TLR7 or HBsAg. Alternatively, antibodies to other liver cell surface markers are used. This assay may be varied in any number of ways and all variations are encompassed by the present invention. This approach enables rapid detection and quantitation of TLR7 or a component in the TLR7 signaling pathway or HBsAg levels.

The subject antibodies are also useful in in situ hybridization analysis such as of biopsy material. Such analysis enables the rapid diagnosis of levels of TLR7 or a component in the TLR7 signaling pathway or HBsAg.

In another embodiment, the method for detection comprises detecting the level of expression in a cell of a polynucleotide encoding TLR7 or a component in the TLR7 signaling pathway or HBsAg. Expression of such a polynucleotide may be determined using any suitable technique. For example, a labeled polynucleotide encoding TLR7 or a component in the TLR7 signaling pathway or HBsAg may be utilized as a probe in a Northern blot of an RNA extract obtained from the cell. Preferably, a nucleic acid extract from the animal is utilized in concert with oligonucleotide primers corresponding to sense and antisense sequences of a polynucleotide encoding the kinase, or flanking sequences thereof, in a nucleic acid amplification reaction such as RT PCR. A variety of automated solid-phase detection techniques are also appropriate. For example, a very large scale immobilized primer arrays (VLSIPS (trademark)) are used for the detection of nucleic acids as, for example, described by Fodor et al. (Science. 251:161-111, 1991) and Kazal et al. (Nature Medicine. 2:153-159, 1996). The above genetic techniques are well known to persons skilled in the art.

For example, for a TLR7- or a component in the TLR7 signaling pathway- or HBsAg-encoding RNA transcript, RNA is isolated from a cellular sample suspected of containing pre-core protein/HBeAg or TLR RNA. RNA can be isolated by methods known in the art, e.g. using TRIZOL (trademark) reagent (GIBCO-BRL/Life Technologies, Gaithersburg, Md.). Oligo-dT, or random-sequence oligonucleotides, as well as sequence-specific
oligonucleotides can be employed as a primer in a reverse transcriptase reaction to prepare first-strand cDNAs from the isolated RNA. Resultant first-strand cDNAs are then amplified with sequence-specific oligonucleotides in PCR reactions to yield an amplified product.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific RNA sequences and cDNA transcribed from total cellular RNA. See generally Mullis et al. (Quant. Biol. 51:263, 1987; Erlich, eds., PCR Technology, Stockton Press, NY, 1989). Thus, amplification of specific nucleic acid sequences by PCR relies upon oligonucleotides or "primers" having conserved nucleotide sequences wherein the conserved sequences are deduced from alignments of related gene or protein sequences, e.g. a sequence comparison of mammalian TLR genes. For example, one primer is prepared which is predicted to anneal to the antisense strand and another primer prepared which is predicted to anneal to the sense strand of a cDNA molecule which encodes a HBsAg or TLR such as TLR7.

To detect the amplified product, the reaction mixture is typically subjected to agarose gel electrophoresis or other convenient separation technique and the relative presence of the TLR7- or component in TLR7 signaling pathway- or HBsAg-specific amplified DNA detected. For example, TLR7 or a component in the TLR7 signaling pathway or HBsAg amplified DNA may be detected using Southern hybridization with a specific oligonucleotide probe or comparing is electrophoretic mobility with DNA standards of known molecular weight. Isolation, purification and characterization of the amplified TLR7 or a component in the TLR7 signaling pathway or HBsAg DNA may be accomplished by excising or eluting the fragment from the gel (for example, see references Lawn et al, Nucleic Acids Res. 2:6103, 1981; Goeddel et al, Nucleic Acids Res. 8:4057-1980), cloning the amplified product into a cloning site of a suitable vector, such as the pCRII vector (Invitrogen), sequencing the cloned insert and comparing the DNA sequence to the known sequence of TLR7 or a component in the TLR7 signaling pathway or HBsAg. The relative amounts of TLR7 or a component in the TLR7 signaling pathway or HBsAg mRNA and cDNA can then be determined.

Real-time PCR is particularly useful in determining transcriptional levels of PCR genes.
Determination of transcriptional activity also includes a measure of potential translational activity based on available mRNA transcripts. Real-time PCR as well as other PCR procedures use a number of chemistries for detection of PCR product including the binding of DNA binding fluorophores, the 5′ endonuclease, adjacent liner and hairpin oligoprobes and the self-fluorescing amplicons. These chemistries and real-time PCR in general are discussed, for example, in Mackay et al, Nucleic Acids Res. 30(6):1292-1305, 2002; Walker, J. Biochem. Mol. Toxicology. 15(3)A21-127, 2001; Lewis et al, J. Pathol. 195:66-71, 2001.

The present invention further provides gene arrays and/or gene chips to screen for the up- or down-regulation of mRNA transcripts. This aspect of the present invention is particularly useful in identifying conditions which result in the down- of HBsAg transcripts or up-regulation of gene transcripts of TLR7 or a component in the TLR7 signaling pathway.

The present invention is further described by the following non-limiting Examples.

**EXAMPLE 1**

*Measurement of TLR7*

*Patients*

In the cross sectional study the patients were classified into three groups based on (a) HBeAg status: HBV HBeAg positive and HBV HBeAg negative and (b) antibody status with concomitant anti-HBc positive and anti-HBs (HBsAg negative). the results are shown in Table 4.

**TABLE 4**

*Demographic data from the three groups of patients with chronic hepatitis B infection tested for toll-like receptors 1-10*

<table>
<thead>
<tr>
<th></th>
<th>Anti-HBc plus anti-HBs positive (n=80)</th>
<th>HBsAg and HBeAg Positive (n=120)</th>
<th>HBsAg positive but HBeAg negative (n=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>49 ± 21</td>
<td>35 ± 18</td>
<td>52 ± 25</td>
</tr>
<tr>
<td>Sex; M:F</td>
<td>52: 28</td>
<td>89 : 31</td>
<td>66: 54</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>27 ± 2</td>
<td>228 ± 21</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>HBV DNA, Log_{10} copies/ml</td>
<td>undetectable</td>
<td>8.06 ± 3.27</td>
<td>3.35 ± 1.26</td>
</tr>
</tbody>
</table>
In the initial longitudinal study patients were treated with PEGASYS (Registered) 180 µg qw + oral placebo or lamivudine qd for 48 weeks (end of treatment) and were followed until 72 weeks. There were 27 patients with HBsAg clearance and 54 patients without HBsAg clearance. PBMCs were collected every 12 weeks for the study period of period.

A summary of the patient information and treatment are shown in Figure 1.

Methods for the detection of TLR-1-9, components of the TLR signal pathway and controls by Real Time PCR

Real time PCR was performed with an ABI PRISM (Registered) 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The samples were incubated for 2 minutes at 50°C and another 10 minutes at 95°C for hot start. The samples were further denatured for 15 seconds at 95°C followed by annealing for 1 minute at 58°C for a total of 40 cycles. The threshold cycle numbers (Ct) were determined with Sequence Detector Software (version 1.6, Applied Biosystems, Foster City, CA, USA) and transformed using the ΔCt or ΔΔCt methods as described by the manufacturer using GADPH as calibrator gene.

Primers for TLR real time PCR are listed (SEQ ID NOs: 1 to 48)

Co-culturing of PBMC from patients with serum containing HBsAg plus HBeAg positive patients

PBMC of the HBsAg plus HBeAg positive patients and anti-HBc plus anti-HBs positive with the serum of HBsAg plus HBeAg positive patients with raised ALT were co-cultured for 7 days. The number of cells used in each system is 1,000,000 while the serum is diluted to give 10,000 copies/ml of HBV DNA by Roche Amplicor assay in RPMI media

HBV specific T cell response

HBV-specific CD4 and CD8+ T-cells were enumerated by IFN-γ producing CD4+ ELISPot assays and tetramer staining for CD8+ T-cells.

Elispot assays: The number of HBV-specific, interferon-γ (IFN-γ) producing CD4 positive T cells will be determined, as described previously [Lau et al. Gastroenterology 2002; 122:614-624]. Briefly, 96-well millilitre plates (Milipore, Bedford, MA, USA) are coated overnight with the primary antibody to human IFN-γ (Mabtech, Nacka, Sweden) at 10 µg/ml
at 4°C. In parallel, PBMC at 2x10⁵/well are cultured in triplicates in RPMI/10% human AB serum with rHBcAg (final concentration 2 µg/ml), rHBsAg (10 µg/ml) and PHA (2 µg/ml) or medium only. After 28 hours the cells are transferred to the coated plates and cultured under the same conditions for 20 hours. The plates are washed and 100 µl of biotin conjugated anti-

IFN-γ antibody (Mabtech, Nacka, Sweden) is added to each well for 2 hours. Next, the plates are washed and incubated for further 2 hours with 100 µl of streptavidin-alkaline phosphatase (Mabtech, Nacka, Sweden). The enzyme reaction is developed with freshly prepared nitro-blue tetrazolium chloride/bromo-chloro-inolyl-phosphate toluidine salt (NBT/BCIP, Roche Diagnostics Ltd., Lewes, UK). The reaction is stopped with distilled water and the spots are counted using ELISPOT reader (ImmunoSpot (Registered) 3B Analyzers from Cellular Technology Ltd, Ohio, USA). The number of specific spot-forming cells (SFC) per 1x10⁶ PBMC is determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells with medium only.

Staining with HLA tetramer complexes: Patients were screened for HLA-A*02 positivity by staining their PBMC with a fluorescent anti-HLA-A2.01 antibody (Serotec, Kidlington, Oxford, UK); selected patients were confirmed to have the HLA-A2.01 allele by PCR DNA typing. The HLA-A*02 tetrameric complexes were purchased commercially (Proimmune, London, UK). The major histocompatibility complex-peptide complexes were formed with two HBV immunodominant epitopes for HBV surface peptide 177.

HBVDNA viral load, cccDNA quantification and HBV genotype

Serum HBV DNA was measured by Roche Amplicor HBV Monitor (Trade Mark) (Cobas) assay. HBV replicative intermediate of the covalently closed circular (ccc) DNA was measured using real time PCR Assay as described by He et al, (Biochem Biophys Res Commun. 2002; 295:1102-7) HBV genotype determined by direct sequencing and comparison to known genotypes.

Real Time PCR for HBVRNA

RNA was extracted from PBMC with QiAmp RNAeasy kit (QiAmp, Valencia, CA, USA) according to the manufacturer's instructions. The concentration of the RNA was quantified by UV cytometry (Beckman DU-600, Beckman Coulter, Fullerton, CA, USA). Total extracted RNA was amplified by real time PCR. Primers and probe for HBV RNA are as follows:
Primers and probe mixture for GAPDH was the product of ABI (Hs00266705).

The reaction mixture was created with the Taqman one-step Master Mix kit (cat no: 4309169, ABI). Briefly, 5 μl of templates RNA was mixed with 1 μl of RNA free water, 10 μl of MASTER MIX, 1 μl of HBV forward and reverse primers and 1 μl of probe, 1 μl of GAPDH primers and 1 μl of probe MIX in a single reaction tube giving a total volume of 20μl. Real time PCR was performed on MJ Chromo qPCR machine (New Jersey, NJ, USA) at 48°C for 30 mins, 95°C for 10 mins, 95°C for 15 sec and finally at 60°C for 1 min for a total of 40 cycles. Relative quantity of the HBV RNA is calculated by the formula of \( \Delta\Delta Ct \).

**EXAMPLE 2**

*Up-regulation of toll-like receptor*

The expression of TLR-1 to 10 were compared by real-time PCR, in PBMCs collected from patients with chronic HBV infection (HBeAg +/-) and patients with resolved HBV infection HBsAg -ve (results for TLR-3, TLR-5, TLR-7 and TLR-9 are shown in Figure 2). The TLR-7 was significantly increased in patients who had resolved HBV (ie., HBsAg -ve, anti-HBs positive). Patients with a natural immunity to HBV (Anti-HBs positive/ HBsAg negative) and HBeAg negative CHB had a significantly higher mRNA expression of TLR7 when compared with patients who were HBeAg positive or HBeAg negative (p=0.02) [Table 5].
TABLE 5
Results of TLR1-10 mRNA expression in T-cells of patients from 3 different stages of chronic hepatitis B infection

<table>
<thead>
<tr>
<th></th>
<th>Anti-HBc positive anti-HBs positive (n=80)</th>
<th>HBsAg positive but HBeAg negative (n=120)</th>
<th>HBsAg and HBeAg Positive (n=120)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>0.53 ± 0.15</td>
<td>0.37 ± 0.13</td>
<td>0.83 ± 0.28</td>
<td>0.35</td>
</tr>
<tr>
<td>TLR2</td>
<td>0.56 ± 0.15</td>
<td>0.77 ± 0.39</td>
<td>0.64 ± 0.12</td>
<td>0.86</td>
</tr>
<tr>
<td>TLR3</td>
<td>1.19 ± 0.32</td>
<td>1.02 ± 0.30</td>
<td>1.04 ± 0.13</td>
<td>0.92</td>
</tr>
<tr>
<td>TLR4</td>
<td>4.55 ± 0.23</td>
<td>3.02 ± 1.53</td>
<td>2.71 ± 0.69</td>
<td>0.76</td>
</tr>
<tr>
<td>TLR5</td>
<td>1.17 ± 0.15</td>
<td>1.38 ± 0.15</td>
<td>1.68 ± 0.32</td>
<td>0.37</td>
</tr>
<tr>
<td>TLR6</td>
<td>0.24 ± 0.04</td>
<td>0.08 ± 0.03</td>
<td>0.33 ± 0.14</td>
<td>0.30</td>
</tr>
<tr>
<td>TLR7</td>
<td>1.56 ± 0.35</td>
<td>0.91 ± 0.16</td>
<td>0.78 ± 0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>TLR8</td>
<td>0.43 ± 0.11</td>
<td>0.34 ± 0.04</td>
<td>0.48 ± 0.10</td>
<td>0.49</td>
</tr>
<tr>
<td>TLR9</td>
<td>0.59 ± 0.07</td>
<td>0.68 ± 0.07</td>
<td>0.63 ± 0.07</td>
<td>0.70</td>
</tr>
<tr>
<td>TLR10</td>
<td>0.48 ± 0.09</td>
<td>0.77 ± 0.32</td>
<td>0.54 ± 0.08</td>
<td>0.59</td>
</tr>
</tbody>
</table>

EXAMPLE 3

Longitudinal Analysis of TLR7 miRNA Expression in T-cells of Patients after Treatment with Pegylated Interferon alfa-2a

Analysis of TLR7 expression by real time PCR was also studied in serial PBMCs collected from patients treated with pegylated interferon (IFN)-α2a at baseline, every 12 weeks during the 48-weeks treatment period and 24 weeks after the end-of-therapy (see Figure 3). The baseline data is presented in Table 6. Again in the longitudinal study there was a significant increase in TLR-7 expression in patients who had cleared HBsAg (HBsAg -ve) versus those who had not cleared HBsAg. This increase in TLR7 expression was detected on week 12 and was persistently increased from week 12 to week 72 in patients who cleared HBsAg.

A total of 9 patients in the HBsAg clearance group developed HBeAg seroconversion at around week 12-24 which was subsequently followed by HBsAg clearance. An increase in TLR7 mRNA expression was observed in all 3 groups of patients at week 12 of treatment with pegylated interferon alfa-2a (Figure 4A). The TLR7 mRNA expression was higher in the HBsAg clearance group than in the HBeAg seroconversion or nonresponder group at week 12 (mean ± SEM 1.5 ± 0.3 vs. 0.7 ± 0.2 vs. 0.5 ± 0.2 respectively, p=0.02).
The peak in TLR7 mRNA expression in the HBsAg clearance group occurred at week 24 (mean ± SEM 1.6 ± 0.4 vs. 0.9 ± 0.2 vs. 0.3 ± 0.1 respectively, p=0.01). This significant increase in TLR7 mRNA expression was maintained until week 72 (p=0.04) (Figure 4 A).

Figure 4B illustrates the cycle threshold (CT) for the housekeeping gene GADPH. There was no significant difference when CT of GADPH at all time points were compared with baseline (all p=NS).

### TABLE 6

Baseline demographic data of patients treated with 48 weeks of pegylated interferon alfa

<table>
<thead>
<tr>
<th></th>
<th>HBsAg clearance (n=9)</th>
<th>HBeAg Seroconverters (n=9)</th>
<th>Nonresponders (n=18)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age; years</td>
<td>41 ± 4</td>
<td>37 ± 2</td>
<td>42 ± 5</td>
<td>0.26</td>
</tr>
<tr>
<td>Sex; M:F</td>
<td>3 : 6</td>
<td>4 : 5</td>
<td>10 : 8</td>
<td>NA</td>
</tr>
<tr>
<td>ALT before treatment; U/L</td>
<td>477 ± 61</td>
<td>397 ± 127</td>
<td>312 ± 247</td>
<td>0.84</td>
</tr>
<tr>
<td>HBV DNA, Log_{10} copies/ml before treatment</td>
<td>8.84 ± 0.23</td>
<td>8.14 ± 0.98</td>
<td>8.33 ± 1.02</td>
<td>0.63</td>
</tr>
<tr>
<td>HBeAg status before treatment:</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>9</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HBeAg status at 6 months after completion of treatment</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>NA</td>
</tr>
<tr>
<td>Anti-HBe at 6 months after completion of treatment</td>
<td>9</td>
<td>18</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>HBV DNA at 6 months after completion of treatment, Log_{10} copies/ml</td>
<td>1.99 ± 1.65</td>
<td>4.92 ± 3.74</td>
<td>6.33 ± 1.61</td>
<td>0.01</td>
</tr>
</tbody>
</table>
EXAMPLE 4

Examination of CD4 and CD8 with TLR7 levels

The HBV-specific CD4 and CD8+ T-cells were enumerated by IFN-γ producing CD4+ ELISPOT assays and tetramer staining for CD8+ T-cells. The CD4 and CD8 levels were correlated to TLR-7 was associated with an increase in HBV specific CD4+ T cells in patients with HBsAg clearance and also an increase in HBV specific CD8+ T cells in patients with HBsAg clearance.

A significant increase in HBV specific interferon-γ producing CD4+ T cells against the core protein was observed in all 2 groups of patients by week 12 of therapy (Figure 4C). The increase in Core-specific CD4+ T cells activity in those with HBsAg clearance was significantly higher at week 24 of therapy (mean ± SEM 256.70 ± 49.22 vs. 181.90 ± 33.23 vs. 91.50 ± 13.21 respectively, p=0.003) when compared with HBeAg seroconversion. This increase in the frequency of interferon-γ producing CD4+ T cells persisted at week 36 (mean ± SEM 243.10 ± 44.98 vs. 138.30 ± 20.82 vs. 86.30 ± 10.78 respectively, p=0.005), week 48 (mean ± SEM 144.00 ± 35.75 vs. 78.89 ± 13.38 vs. 44.50 ± 9.44 respectively, p=0.02), week 60 (mean ± SEM 89.20 ± 21.53 vs. 43.90 ± 11.94 vs. 31.20 ± 11.89 respectively, p=0.05) and week 72 (mean ± SEM 77.40 ± 24.76 vs. 47.90 ± 12.73 vs. 30.50 ± 7.65 respectively, p=0.04) (Figure 4C).

No significant difference could be detected in the Surface-specific CD4+ T cells activity between the 2 groups (all p=NS) (Figure 4D).

HBV specific CD8+ response was also analyzed in parallel in 13 HLA-A2 patients (32.1%); 3 with HBsAg clearance, 4 with HBeAg seroconversion and 6 nonresponder; with ELISpot to Core 18-27 peptide and Surface 177-185 peptide. Enhancement in interferon-γ production to Core 18-27 peptide was detected in all 3 groups beginning at week 24 of treatment. A peak in interferon-γ production to Core 18-27 peptide occurred at week 24 of treatment. Patients with HBsAg clearance had a significantly higher frequency HBV specific interferon-γ producing CD8+ T cells by ELISpot to Core 18-27 peptide at week 36 (mean ± SEM 189.50 ± 39.24 vs. 95.00 ± 28.32 vs. 56.50 ± 7.45 respectively, p=0.02), 48 (mean ± SEM 175.00 ± 30.92 vs. 84.50 ± 20.12 vs. 47.00 ± 9.22 respectively, p=0.03), 60 (100.50 ± 20.15 vs. 40 ± 5.50 vs. 11 ± 2.01 respectively, p=0.04) and week 72 (80.00 ± 13.43 vs. 40.50 ± 5.50 vs. 23.50 ± 4.23
respectively, p=0.05) of therapy when compared with the 4 HLA-A2 patients with HBeAg seroconversion and 6 HLA-A2 positive nonresponders (Figure 4E).

There was no difference in interferon-γ production to Surface peptide from CD8+ T-cells.

This increase in interferon-γ production to Core peptide from CD8+ T-cells was associated with an increase in serum ALT levels more than 2 times the upper limit of normal and occurred after HBsAg clearance was detected. The increase in both HBV specific CD4+ and CD8+ T cell activity were preceded by an increase in TLR7 mRNA expression. The clinical features of a typical HLA-A2 patient with HBsAg clearance is shown in Figure 4F.

Other viral markers such as serum HBV DNA as measured by Roche Amplicor HBV Monitor (Trade Mark) (Cobas) assay and HBV genotype determined by direct sequencing, and ALT were also determined.

EXAMPLE 5

**TLR-7 expression in an ex-vivo co-culture model**

Live HBV wild-type virus was used to infect the PBMCs of patients with (n=30) and without HBsAg clearance (n=30). The levels of TLR-7 were measured by real-time PCR in a time course experiment (Figure 5). There was a significant increase in TLR-7 in patients who were HBsAg -ve versus HBsAg +ve.

A TLR-7 agonist was added to the ex vivo co-cultured cells and the TLR-7 was measured by real-time PCR and western blot (Figure 6). In the presence of the TLR-7 agonist 2 ng/µl there was an increase in TLR-7 RNA and protein expression in HBsAg +ve cells by 50% (Figure 7).

EXAMPLE 6

**HBV replication in the presence of a TLR-7 agonist**

The levels of HBV RNA (Figure 8) and cccDNA (Figure 10) were examined after 7 days using the PBMc’s ex vivo co-cultured cells in the presence of the TLR-7 agonist. The HBV RNA levels in PMBCs from patients that were HBsAg +ve increased over the 7 days. HBV RNA levels in PBMCs from patients that were HBsAg +ve treated with the TLR-7 agonist
were lower similar to the levels found in PBMCs from patients that were HBsAg -ve. Figure 9 shows the specificity of the HBV RNA detection using a Northern analysis. The HBV RNA decreased on Northern Blotting after inhibition of HBV by SiRNA according to the methods described by Wu et. al. Gastroenterology 2005; 128:708-716).

The HBV RNA positive controls (marked Control +), was extracted RNA from liver tissue of HBsAg+/HBeAg+/ALT> 3 times the upper limit of normal (range 13-51 LVL) patient with HBV DNA>1,000,000 copies/ml by the Amplicor assay while negative controls (Control -) contain the reaction mixture but no nucleic acids.

Similarly, the HBV cccDNA levels in PMBCs from patients that were HBsAg +ve increased over the 7 days. HBV cccDNA levels in PMBCs from patients that were HBsAg +ve treated with the TLR-7 agonist were lower similar to the levels found in PMBCs from patients that were HBs Ag -ve. For cccDNA as well, the positive control (Control +) is DNA extracted from the liver tissue of an HBsAg+/HBeAg+/ALT> 3 times the upper limit of normal (range 13-51 LVL) patient with HBV DNA>1,000,000 copies/ml by the Amplicor assay. The negative control (Control -) is the reaction mixture without nucleic acid.

In a time course after 7 days HBV DNA levels from patients that were HBsAg +ve increased (Figure 11). HBV DNA levels in PMBCs from patients that were HBsAg +ve treated with the TLR-7 agonist were lower similar to the levels found in PMBCs from patients that were HBsAg -ve.

EXAMPLE 7

HBV T cell response in the presence of a TLR-7 agonist

The HBV CD4 and CD8 levels for both core and envelope epitopes in PMBCs isolated from patients that were HBsAg +ve were lower relative to the HBV CD4 and CD8 levels for both core and envelope epitopes in PMBCs from patients that were HBsAg +ve treated with the TLR-7 agonist, which in turn were similar to the levels found in PMBCs from patients that were HBsAg -ve (Figure 10).
EXAMPLE 8

**Effect of a TLR-7 agonist in the ex vivo co-culture model on components TLR signal pathway response.**

Levels of the different known components of TLR signalling pathway were measure in the presence of the TLR-7 agonist. There were differential effects on the MyD88-dependent pathway, which was studied with real-time PCR (Figure 12).

In *ex vivo* model, the higher TLR7 expression in PBMCs from patients with HBsAg clearance is accompanied by upregulation of MyD-88, TRAP6 and NF\(\iota\) expression.

Hence, up-regulation of TLR-7 plays an essential role in serological clearance of HBsAg in patients with chronic HBV infection. The activation of adaptive immune response by TLR-7 is mediated by MyD88-dependent pathway.

EXAMPLE 9

**Effect of HBV on TLR7 Expression on T-cells**

To examine the functional specificity of TLR7, isolated T-cells of HBV immune and HBV positive groups were incubated with WtHBV in the presence of autologous APCs for 168 hours. It was found that incubation with wtHBV resulted in suppression of TLR7 mRNA expression on the T-cells within 12 hours in both groups. However, the TLR7 mRNA expression in HBV immune group started to recover by 24 hours of incubation while in the HBV positive group, TLR7 mRNA expression remain persistently suppressed until 168 hours (Figure 13A).

The TLR7 mRNA expression in the HBV positive group was significantly lower at 168 hours (mean ± SEM 0.10 ± 0.04) of incubation when compared with baseline (mean ± SEM 0.28 ± 0.03, p=0.03). However, no significant difference in the TLR7 mRNA expression in patients with natural immunity to HBV when 168 hours of incubation was compared with baseline (mean ± SEM 0.87 ± 0.14 vs. 0.84 ± 0.19, p=NS).
Recovery of TLR7 is Associated with an Increased Interferon-γ Production from CD4+ and CD8+ cells

Interferon-γ production was tested from CD4+ and CD8+ T-cells after incubation with wtHBV by ELISpot. In HBV immune group, interferon-γ production from CD4+ T cells by ELISpot started to increase at 24 hours and peaked at 168 hours. This increase in interferon-γ production from CD4+ T cells corresponded to the increase in TLR7 mRNA expression in the T-cells. The interferon-γ production from CD4+ T cells was higher in the HBV immune group at 168 hours of incubation (Figure 13B).

On the other hand, in the HBV positive group, interferon-γ production from CD4+ T-cells only started to increase at 48 hours but the increase in interferon-γ production from CD4+ T-cells in the HBV positive group was significantly lower than in the natural immunity to HBV group.

The increase in interferon-γ production from CD4+ cells in the HBV immune group also translated to increased interferon-γ production from CD8+ T-cells (Figure 13C). Interferon-γ production from CD8+ T-cells in the HBV immune group started to increase at 72 hours and similar to interferon-γ production from C48+ T-cells, peaked at 168 hours (Figure 13C).

Interferon-γ production from CD8+ T-cells in the HBV positive group started to increase at 72 hours but was significantly lower than in the HBV immune group (Figure 13C).

TLR7 Knockdown with siRNA Inhibits Interferon-γ Release in Patients with Natural Immunity to HBV

The effect of siRNA-mediated knockdown of TLR7 on interferon-γ release was determined. RNAi for TLR7 resulted in a > 95% knockdown, which persisted for 7 days (Figure 13A). The siRNA had no effect on GADPH (Figure 13B). Knockdown of TLR7 RNAi resulted in decreased interferon-γ production from both CD4+ and CD8+ T cells in the HBV immune group (Figure 13C and Figure 13D).
EXAMPLE 12

**TLR7 Agonist Enhanced CD4+ and CD8+ T-cells Response in HBV Infected Patients**

Treatment with 2 and 5 ng/ml TLR7 agonist resulted in a significant enhancement in interferon-γ producing CD4+ T-cells even in HBV positive group at 24 hours after incubation with wtHBV (Figure 14A). The enhancement in CD4+ T-cells activity peaked at 168 hours of incubation.

The enhancement in CD4+ T-cells activity with TLR7 agonist was translated into enhancement of CD8+ T-cells activity in HBV positive patients. The enhancement in CD8+ T-cells activity occurred at 120 hours and peaked at 168 hours (Figure 14B).

EXAMPLE 13

**Action of TLR7 on T-Cells Can be Enhanced by Interferon-α/β**

In order to determine whether enhancement of T-cells by TLR7 is cytokine mediated, the incubated isolated T-cells from HBV positive group with wtHBV in the presence of interferon-α antibody (Ab) and interferon-β Ab for 168 hours (peak of interferon-γ production as observed in Figure 13C).

The enhancement in interferon-γ release from CD4+ T-cells of HBV Positive patients by TLR7 agonist 2.0 ng/ml in *in vitro* culture was significantly reduced when both interferon-α and interferon-β Ab was added into the *in vitro* culture (Figure 15A). The addition of either interferon-α Ab or interferon-β Ab independently into the *in vitro* culture did not result in a significant reduction in the interferon-γ release from CD4+ T-cells of HBV Positive patients by TLR7 agonist 2.0 ng/ml (Figure 15A). TLR7 agonist at 2.0 ng/ml was selected for this experiment as this was the optimum dose for enhancement of interferon-γ production from CD4+ T-cells (Figure 14A and Figure 14B).

EXAMPLE 14

**TLR7 Agonist Further Enhances T-cells Activation Through Interferon-α/β Secretion by DCs**

In order to determine the source of the interferon-α/β (type I interferon) secretion, focus was made on NK cells and DCs from HBV positive group. DCs from HBV positive group were
cultured for 24 hours in flat-bottom plates with and without TLR7 agonist. Interferon-α/β (type I interferon) activity in the supernatant were measured by bioassay. As shown in Figure 15B, the bioactive interferon produced by DCs appeared to be mostly interferon-α/β (type I interferon).

NK cells were also isolated from the T-cells of HBV positive group. The bioactive interferon produced by NK cells were mostly interferon-γ release.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.
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CLAIMS:

1. A method for monitoring acute, chronic or persistent infection by HBV during or subsequent to treatment in a subject, said method comprising; determining changes in levels of TLR7 or a component in the TLR7-signaling pathway during or subsequent to treatment wherein elevated levels of TLR7 is indicative of improved potential for HBsAG clearance and resolution of HBV infection.

2. The method of Claim 1 wherein the subject is a human.

3. The method of Claim 2 wherein the human subject has chronic HBV infection.

4. The method of Claim 1 wherein the treatment is the administration of an agent which up-regulates the level or activity of TLR7 or a component in the TLR7-signaling pathway.

5. The method of Claim 4 wherein the treatment further comprising the administration of a nucleoside or nucleotide analog, anti-viral agent or an immunomodulatory agent.

6. The method of Claim 5 wherein the immunomodulatory agent is IFNγ.

7. A method for monitoring acute, chronic or persistent infection by HBV in a subject said method comprising determining the levels or activity of TLR7 or a component in the TLR7 signaling pathway wherein an elevated level or activity of TLR7 is indicative of improved capacity of the subject to induce clearance of HBsAg, to generate anti-HBsAg antibodies and/or to facilitate resolution of HBV infection.

8. The method of Claim 7 wherein the subject is a human.

9. A method for monitoring a response to a therapeutic protocol directed against acute, chronic or persistent infection by HBV in a subject said method comprising determining the level or activity of a TLR7 or a component of the TLR7 signaling pathway wherein an elevated level or activity of TLR7 or component of the TLR7 signaling pathway is indicative of an improved capacity of the subject to resolve HBV infection.
10. The method of Claim 9 wherein the therapeutic protocol comprises the administration of an anti-HBV agent selected from a nucleoside or nucleotide analog capable of inhibiting HBV DNA polymerase, an antagonist of HBV DNA polymerase, a cytokine and an antigen capable of stimulating an immune response to HBV and/or a component thereof.

11. The method of Claim 9 or 10 wherein the subject is a human.

12. The method of Claim 11 wherein the HBV infection is chronic.

13. A method of treating a subject infected with HBV said method comprising administering to said subject an effective amount of an agent which up-regulates TLR7 or a component of the TLR7 signaling pathway.

14. The method of Claim 13 wherein the subject is a human.

15. The method of Claim 14 further comprising the administration of a nucleoside or nucleotide analog, anti-viral agent or an immunomodulatory agent.

16. The method of Claim 15 wherein the immunomodulatory agent is IFNγ.

17. A composition comprising an agent which up-regulates TLR7 or a compound in the TLR7-signaling pathway and one or more pharmaceutically acceptable carriers, diluents and/or excipients.

18. The composition of Claim 17 further comprising one or more of a nucleoside or nucleotide analog, an anti-viral agent or an immunomodulatory agent.

19. The composition of Claim 18 wherein the immunomodulatory agent is IFNγ.

20. Use of an agent which up-regulates TLR7 or a component of the TLR7 signaling pathway alone or in combination with an anti-HBV agent in the manufacture of a medicament for the treatment of HBV infection in a subject.

21. Use of Claim 20 wherein the anti-HBV agent is selected from a nucleoside or
nucleotide analog capable of inhibiting HBV DNA polymerase, an antagonist of HBV DNA polymerase, a cytokine and an antigen capable of stimulating an immune response to HBV and/or a component thereof.

22. Use of Claim 20 or 21 wherein the subject is a human.

23. Use of Claim 22 wherein the human has chronic HBV infection.
Methods

Cross sectional
- 60 HBeAg+/ 60 HBeAg-
- 40 anti-HBc and anti-HBs +

Longitudinal
- 27 patients with HBsAg clearance
- 54 matched patients without HBsAg clearance

PEGASYS® 180 μg qw + oral placebo/lamivudine qd

PBMCs collected every 12 weekly during study period

Marcellin NEJM 2004
Lau NEJM 2005

Figure 1
Results: TLR Expression in PBMC

![Graph showing TLR expression in PBMC with HBSAg, HBeAg, and HBe- groups.](image)

Figure 2
TLR7 During Pegylated IFN

Baseline
HBsAg Clearance
HBsAg+
Demographics

HBeAg:
+/- 10/17 20/34

Figure 3
Figure 4C

Frequency of HBV Core antigen specific interferon-γ producing CD4+ T Cell

Key:
- □ HBsAg Clearance
- □ HBsAg Positive
- □ Nonresponder

Time of Therapy (Weeks)
- BL
- wk12
- 24
- Wk 36
- Wk 48
- Wk 60
- Wk 72

SFC/10^6 PBMC
- 0
- 50
- 100
- 150
- 200
- 250
- 300
- 350
Figure 4D

Frequency of HBV surface antigen specific interferon-γ producing CD4+ T cell
SFC/10^6 PBMC

Time of Therapy (Weeks)

- □ HBsAg Clearance
- □ HBeAg Seroconversion
- □ Nonresponder

Frequency of Core peptide 18-27 interferon-γ producing CD8+ T cells

* P=0.02
* P=0.03
* P=0.02
* P=0.05

* When 3 groups were compared

Figure 4E
Figure 4F
Dynamic Time Course Suppression of TLR7 Expression in HBsAg+ Patients Compared to Anti-HBc+/anti-HBs+ in Co-culture Model

![Graph showing time course suppression of TLR7 expression](image)

- HBsAg Clearance
- HBsAg Positive

$P=0.01$

+ Performed with 30 patients each group

**Figure 5**
Use of TLR7 Agonist

TLR7 on Real Time PCR
with TLR7 Agonist 1 μg/ml

TLR7 on Western Blot (densitometry)
With TLR7 Agonist 1 μg/ml

A(i)  
Relative expression
(TLR7/GAPDH)

A(ii)

HBsAg+/TLR7  
HBsAg+

B
MTT Assay

Absorbance (%)

All p=NS

HBsAg+
Control
HBsAg+/TLR7

Day 0  
Day 7

Figure 6
HBV RNA is Increased After Co-culture

![Graph showing HBV RNA levels at Day 0 and Day 7 with statistical significance](image)

Figure 7
Specificity of HBV RNA Detection

Day 7 co-culture in Untreated SiRNA HBVSI+  

- On Day 7 co-culture, SiRNA used to target HBV
- On Northern Blot, 2.1/2.4 Kb inhibited decreasing HBV replication intermediate

2.1/2.4 Kb

GADPH

+Wu Gastroenterology 2005

Figure 8
cccDNA in PBMCs After Co-culture with HBV

Figure 9

HBsAg-
HBsAg+ (TLR7)
Control-
Control+
Figure 10

CD4 Response

CD8 Response

Experiments repeated in 10
Pathways Upregulated at mRNA Level on Day-7 Co-culture

Figure 12
Figure 13
Figure 15

(A) T-Cells isolated from HBsAg plus HBcAg positive patients incubated with vHBV
Frequency of interferon-γ production from CD4+ T-cells determined by ELISpot assay.

(B) Interferon-γ in Supernatant (U/mL)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Interferon-γ (U/mL)</th>
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<tbody>
<tr>
<td>HBV Positive DCs without TLR7 Agonist</td>
<td>1000</td>
</tr>
<tr>
<td>HBV Positive DCs plus TLR7 Agonist</td>
<td>3000</td>
</tr>
<tr>
<td>HBV Positive DCs plus TLR7 Agonist 2.0 ng/ml</td>
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**Oligonucleotide primers and probes used for Taqman analysis**

<table>
<thead>
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<th>Forward</th>
<th>Probe</th>
<th>Reverse</th>
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**Figure 16**
Diagnostic and therapeutic methods and agents

US 60/735480
2005-11-09

PatentIn version 3.2

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forward oligonucleotide primer

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3
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4
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