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The use of DNA for the development of novel prophylactic and therapeutic vaccines against the hepatitis B virus.

By

Paul J Payette, BSc, MSc

A thesis submitted in partial fulfillment of the requirements for the degree

of

Doctor of Philosophy

University of Ottawa

2001

Abstract

The prevention and control of new and existing hepatitis B virus (HBV) infections remains an important global health concern. Despite current global immunization efforts, new HBV infection continues to occur. It is estimated that about 350 million people worldwide are currently afflicted by viral persistence. This body of work explores the possibility of developing improved prophylactic and possibly therapeutic vaccines against HBV using DNA technology. It has become well established that the effective control of HBV infection requires the development of strong humoral and cell mediated immunity (CMI) including the production of the pro-inflammatory cytokines interferon (IFN) γ and tumor necrosis factor (TNF) α that are associated with a CMI response. In the absence of these responses infection is not resolved and persistence ensues. The ability of DNA (either in the form of a DNA vaccine or as immunostimulatory DNA sequences in combination with protein antigens) to stimulate both humoral and cellular immune responses that include the production of IFNγ and TNFα, makes them attractive candidates for development of novel prophylactic and therapeutic agents in the struggle against HBV infection. This work demonstrates that immunization strategies that include DNA technology were capable of controlling HBV gene expression in a hepatitis B surface antigen transgenic mouse model as well as provide protection against infectious HBV challenge in chimpanzees. The quality of the immune responses induced in the chimpanzees suggests that the therapeutic potential of these immunization strategies observed in the mouse model may also extend to higher primates.

Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Heather L. Davis. Her faith, guidance, and support have allowed me to become a better scientist.

I would like to thank the University of Ottawa, Medical Research Council of Canada, the government of Ontario, and Coley Pharmaceutical Group Inc. for their support of this work.

I would like to thank our collaborators, Dr. Marie-Louise Michel of the Pasteur Institute, and Dr. Robert Purcell of the National Institute of Health for the wonderful opportunities and experiences.

I would like to thank Dr. R Bradbury, Max Shapiro and their team at Bioqual Inc. for their expertise and assistance with the chimpanzee immunizations.

I would like to thank the members of my thesis advisory committee, Dr. Kathryn Wright, Dr. Ken Dimock, and Dr. Ken Rosenthal, for their valuable and much appreciated input.

I would like to thank my colleagues, Dr. Risini D Weeratna, Dr. Michael J McCluskie, and the whole research team at Coley Pharmaceutical Group Inc. for continual encouragement and support.

I would like to thank the staff of the animal care facility at the LOEB Health Research Institute for their guidance and support.

I would like to thank Dr. Wallace Lauzon for our continual and stimulating immunological discussions.

I would like to thank my parents for giving me the drive to succeed.

Finally I would like to thank my wife Genny Giroux, whose love and support have been, and will continue to be, the foundation of my career. She is my partner, my confidant, and most of all, my very best friend.

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List of Abbreviations

β-gal	β-galactosidase
β–2ME	β-2 mercaptoethanol
ADCC	antibody-dependent cellular cytotoxicity
alum	aluminum hydroxide
anti-HBc	anti-HBcAg antibodies
anti-HBs	anti-HBsAg antibodies
APC	antigen presenting cell
C1D	MHC class I deficient
C2D	MHC class II deficient
CCAC	Canadian Council on Animal Care
cccDNA	Covalently closed circular DNA
CFA	Complete Freunds' Adjuvant
CID_{50}	chimp infectious dose 50
CMI	cell mediated immunity/cell mediated immune
ConA	concanavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DNA-PK	DNA dependent protein kinase
EBV	Epstein Barr virus
ELISA	enzyme linked immunosorbant assay
ER	endoplasmic reticulum
ERK	extracellular signal regulated
FBS	fetal bovine serum
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B pre-core antigen
HBsAg	hepatitis B surface antigen (collectively S1.S2.S, S2.S and S)
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HRP	horseradish peroxidase
IFN	Interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IL-1R	IL-1 receptor
IM	intra-muscular
IP	intraperitoneal
IV	intravenous
JNK	c-Jun *N*-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MHC	major histocompatability complex
MOI	multiplicity of infection
MyD88	myeloid differentiation marker 88

NK	natural killer
NMS	normal mouse serum
ODN	oligodeoxynucleotide
ORF	open reading frame
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cell
Pfn K/O	perforin knock-out
pg	pre-genomic
PHA	phytohemagglutinin
PRR	Pattern recognition receptors
RNP	ribonucleoprotein
ROS	reactive oxygen specie
rVV-S	recombinant vaccinia virus coding for the HBV S protein
S	small hepatitis B envelope protein
S1.S2.S	large Hepatitis B envelope protein
S2.S	middle hepatitis B envelope protein
sALT	serum alinine aminotransferase
SAPK	stress activated protein kinase
SCID	severe combined immunodeficient
SI	stimulation index
TA	*tibialis anterior*
Th1	T helper 1
Th2	T helper 2
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor associated factor
VV-WT	wildtype vaccinia virus

1. Introduction

1.1. History of hepatitis B

The clinical outcomes of viral hepatitis, most notably jaundice, have been documented throughout history and date back as early as the fifth century BC when Hippocrates described the occurrence of epidemic jaundice (1). However, it wasn't until 1883 that Lurman in Germany first documented, during a smallpox immunization campaign with a vaccine prepared from human lymph, that a form of hepatitis could be transmitted following the direct inoculation of human blood products (1).

During World War II, epidemics of hepatitis were of particular significance in military personnel who were recipients of a yellow fever vaccine that had been stabilized with pooled human serum (1, 2). Further documentation of hepatitis transmission following therapy with blood products, coupled with the identification of hepatitis outbreaks associated with high risk groups and unrelated to blood exposure, was the impetus for controlled studies on hepatitis transmission (1). Human volunteer studies throughout the 1930s and 1940s implicated a viral association with hepatitis with two possible etiological agents that had two different modes of transmission (1).

MaCallum and Bauer introduced the term "hepatitis B" in 1947 to distinguish what was known at the time as "homologous serum" hepatitis from "infectious hepatitis", which they designated as hepatitis A (1, 2). It was understood that the epidemiology of these two diseases was very different. Hepatitis A, which

appeared to occur primarily in children, was transmitted by the fecal-oral route and had an incubation time of 2-6 weeks whereas hepatitis B that appeared to occur primarily in adults, was transmitted by exposure to blood products and had a longer incubation time of 2 to 6 months.

Saul Krugman and his colleagues later confirmed the existence of two separate etiological agents for hepatitis in their work at the Willobrook Institute in the 1960s and 70s (3-5). Initially designated as MS-1 and MS-2, after the patient they were isolated from, these agents resembled the hepatitis A and B designations, respectively, previously established by MacCallum and Bauer. Krugman and his colleagues also demonstrated the existence of homologous immunity generated in response to each of these agents (1).

Also in the early 1960s, but unrelated to Krugman's studies, Baruch Blumberg made his own serendipitous but none the less landmark discovery. While studying the genetic polymorphism of serum proteins at the National Institute of Health (NIH), he discovered a novel protein found in the serum of an Australian aborigine (6). Identified as the Australian antigen, it was the first of the hepatitis B virus (HBV) proteins to be described and was subsequently linked to acute hepatitis B (1). Discovery of the Australian antigen in turn led to the development of the first HBV-specific diagnostic tools (7, 8).

The first visualization of virus like particles associated with individuals that were positive for the Australian antigen came in 1970 when David Dane and his colleagues, using electron microscopy, first identified the 42 nm particles that came to be known as "Dane particles" (9). The 42 nm particles proved to be

hepatitis B virions and the "Australian antigen" came to be known as the hepatitis B surface antigen (HBsAg), to underline its position on the viral envelope (1, 2).

Krugman and his colleagues subsequently described that viral infectivity could be destroyed by heating and that heat inactivated virus retained some antigenicity that was capable of inducing partial protection when given to volunteers who were subsequently challenged with his HBV strain MS-2 (10, 11). Krugman also demonstrated that hepatitis B immune globulin was also capable of preventing or modifying the course of HBV infection (12).

Finally in 1981, following the extensive characterization of the HBsAg and a clinical trial directed by Wolf Szmuness and Cladd Stevens (13), the first HBV vaccine, which was composed of HBsAg derived from the plasma of chronically infected humans, was licensed. However, based on perceived safety concerns related to the use of human blood products, the plasma-derived vaccine was replaced by the first yeast derived recombinant HBsAg vaccine in 1986.

1.2. Epidemiology of Hepatitis B

Although monumental advances have been made in HBV research and these have led to the development of effective prophylactic vaccines, infection by HBV remains an important global concern. More than 2 billion people worldwide have been infected by HBV, with 350 million developing a persistent/chronic infection (14). Chronic HBV infection is associated with an increased risk of developing liver cirrhosis and hepatocellular carcinoma (HCC), with the risk of HCC increasing 100 fold (15).

The global distribution of chronic HBV infection varies. Approximately 45% of the world's population lives in highly endemic areas (prevalence of ≥8%), 43% of the population lives in areas of intermediate endemicity (prevalence of 2-7%) and 12% live in low endemic areas (prevalence of <2%) (14).

HBV transmission occurs primarily by percutaneous and mucous membrane exposure to contaminated blood or body fluids (14, 16) and the major modes of transmission include, intravenous (IV) injection drug use, sexual or household contact with infected persons, perinatal transmission from mother to infant and nosocomial exposure (16). The importance of a given mode of transmission is tightly related to the level of endemicity in a given area (14, 16). In highly endemic areas HBV is primarily transmitted perinatally or soon after birth in early childhood. Whereas in low endemic areas, transmission is generally restricted to adults and occurs primarily by sexual contact or other high risk behaviors such as IV drug use.

1.3. Hepatitis B Virology

HBV is a non-cytopathic enveloped DNA virus that belongs to the family of closely related viruses called *hepadnaviridae* (17, 18). These viruses, which also include woodchuck hepatitis virus, ground squirrel hepatitis, and duck hepatitis B virus, share key features that distinguish them from other DNA viruses. These include: enveloped virions that contain a relaxed circular partially double stranded DNA genome approx. 3kb in size, virion-associated viral polymerase responsible for genomic DNA synthesis during late replication steps, excessive production of viral membrane proteins resulting in the release of sub-viral

particles, narrow host range, and development of persistent infections with pronounced hepatotropism (17).

As mentioned earlier, the infectious hepatitis B virion consists of a 42 nm viral particle first described by Dane et al. in 1970 (9) and subsequently named the Dane particle. As with all hepadnaviruses, the Dane particle consists of an enveloped nucleocapsid containing the partially double stranded circular DNA genome associated with a viral polymerase (17, 18). The viral envelope consists of host derived plasma membrane in which is anchored, to differing degrees, the three viral envelope or surface (S) proteins; the large, middle and major (sometimes called small), which are collectively referred to as HBsAg. As the name suggests the major S protein makes up the majority of HBsAg present in the viral envelope, followed by the middle, then large S proteins.

The HBV genome consists of a full-length 3.2 kb negative sense and an incomplete positive sense DNA molecule that encodes four overlapping transcripts of 3.5, 2.4, 2.1, and 0.7 kb in length (17, 18). Four independent promoters and two enhancers regulate viral gene transcription and all viral transcripts terminate at a single common polyadenylation signal (17, 18). The transcriptional enhancers demonstrate a strong influence over HBV gene expression and appear to play an important role in the apparent hepatotropism of the virus (19).

The 3.5 kb viral transcript contains two open reading frames (ORFs), the nucleocapsid or core ORF and the polymerase ORF, and also serves as the

genomic template during viral replication, which is regulated by the core polymerase promoter.

The nucleocapsid or core ORF codes for the HBV core protein (HBcAg), which self assembles into the HBV nucleocapsid particles that encapsidate the viral genome. Due to the heterogeneous use of alternate transcriptional start sites at the 5' end of the 3.5 kb transcript, a modified form of the HBcAg is also produced that contains an additional amino acid sequence at its amino terminus known as the pre-core. This protein is targeted, by virtue of a signal sequence within the pre-core region, to a secretory pathway that generates 3' and 5' truncations and produces the circulating protein referred to as the HBV e antigen (HBeAg). Although the function of HBeAg remains unclear, its presence serves as a marker for active viral replication and almost always co-exists with circulating HBV DNA. It has been postulated that circulating HBeAg may play a role in the development of viral persistence through modulation of immune responses directed towards HBV (20).

The polymerase ORF encodes the viral polymerase, which is necessary during the final steps of viral replication when the pre-genomic 3.5 kb viral mRNA is reverse transcribed into the partially double stranded DNA genome that is encapsidated into the self-assembled HBV nucleocapsids.

The 2.4 and 2.1 kb transcripts contain the ORFs that encode the large (S1.S2.S) and the middle (S2.S)/small (S) envelope proteins respectively. As with the 3.5 kb transcript, the heterogeneous use of alternate translational start sites

accounts for the differential production of two proteins from the single 2.1 kb transcript.

Finally the 0.7 kb viral transcript contains an ORF that encodes the HBV X protein. As with HBeAg, the exact function of the X protein remains unclear, although it has been linked to DNA binding independent transcriptional transactivation, signal transduction as well as influencing the development of HCC.

HBV replication, like all hepadnaviruses, is unique among DNA viruses in that it does not proceed via the semi-conservative replication of DNA molecules. Rather, genomic replication is achieved by the reverse transcription of the 3.5 kb RNA intermediate, which serves as a genomic template, by the viral polymerase (21).

The first stage of viral replication is attachment and entry into the host cell, and it is clear that the viral envelope proteins of HBV are involved since antibodies to these proteins can block this process (22, 23). However, it remains unclear what the cellular targets of viral attachment are, and exactly how each protein contributes to this process. The nucleocapsid then moves to the nuclear membrane where the genomic DNA is delivered in to the nucleus by an as yet unidentified mechanism that is thought to be nucleocapsid independent (24). Once inside the nucleus, the relaxed partially double stranded DNA genome is repaired by the host DNA repair machinery (25) and converted to a covalently closed circular or cccDNA molecule. It is this form of the HBV genome that serves as the transcriptional template for the different viral mRNAs.

As viral transcripts are generated, viral proteins begin to be translated by the host's transcriptional machinery (17). Core proteins begin to self-assemble into nucleocapsid particles ready to receive their genomic cargo, and the envelope proteins are inserted into the membranes of the endoplasmic reticulum in preparation for viral budding (17). Surplus envelope protein is generated during replication such that 20 nm non-infectious sub viral particles consisting of only plasma membrane and HBsAg and devoid of genetic material are constitutively released by the host cell and readily detectable in the circulation (17). As replication proceeds, the newly translated viral polymerase associates, in a sequence-specific fashion, to the 3.5 kb pre-genomic (pg) RNA transcript (26, 27). Together they form a ribonucleoprotein (RNP) complex that is targeted for encapsidation by host derived chaperone proteins (28, 29). Once encapsidated, the viral polymerase, which has been activated by its interaction with the pgRNA (30), initiates a complex reverse transcription process that generates the partially double stranded HBV genome (31-34).

The next step in viral replication is the acquisition of the viral envelope via budding into the endoplasmic reticulum (ER) (17). This process is tightly regulated by genomic DNA maturation since nucleocapsids without DNA are never released from the cell (35, 36). Viral budding is mediated in part by the interaction of competent nucleocapsids with S1.S2.S proteins inserted in the ER membrane (18) and may be influenced by conformational changes that occur in the nucleocapsid proteins during DNA maturation (37-39). After budding into the

ER, the newly formed infectious virions or Dane particles are released from the cell through the Golgi apparatus (40).

1.4. Kinetics of HBV infection

HBV infection proceeds in four distinct stages (41). The initial or *early replicative* stage (2-4 weeks) is characterized by immune tolerance with high levels of circulating HBsAg, HBeAg and HBV DNA and normal liver function. The only detectable immune responses at this stage are antibodies to HBcAg (anti-HBc), but these fail to clear infection. The second or *late replicative* stage (3-4 weeks) is characterized by active hepatitis where virally infected hepatocytes are eliminated by HBV-specific immune responses, and this results in elevated liver enzymes. The third or *early integrative* stage is characterized by viral clearance, the appearance of anti-HBe antibodies, the loss of circulating HBeAg and HBV DNA and the normalization of liver function. The final or *late integrative* stage is characterized by complete immunity as indicated by the disappearance of circulating HBsAg and concomitant development of anti-HBsAg (anti-HBs) antibodies, which provide long-term protection against subsequent infection.

The vast majority of adults who become infected with HBV are able to clear the virus. This is usually in the absence of any severe clinical manifestations, except for rare cases (1-2%) when an excessive immune response to HBV infection in adults may lead to the development of fulminate hepatitis that may be severe enough to result in death (41, 42). Unfortunately in five to ten percent of adult and 90-95% of neonatal transmissions, the virus is not cleared, resulting in persistent infection and the possible development of chronic hepatitis and liver

disease. Persons infected early in life generally remain in the *early replicative* stage, where immune tolerance prevails, and since active hepatitis is absent, they are classified as asymptomatic or "healthy" chronic carriers. Persistent infections that arise later in life generally remain in the *late replicative* stage, where active hepatitis prevails, and this results in the development of chronic active hepatitis with a significantly increased risk of developing liver cirrhosis and HCC (42). This is an important health concern in endemic regions of the world where the majority of HBV infection is acquired neonatally, and leads to the development of large populations of persistently infected individuals, which in turn constitute enormous reservoirs of infectious virus. Many of these individuals go on to develop and succumb to the secondary sequelae associated with chronic liver disease, placing a heavy burden on health care systems. Currently, it is estimated that more than 350 million people worldwide are persistently infected with HBV (41), for whom there is no truly effective therapy.

1.5. Immunology of HBV Infection

HBV is described as a non-cytopathic virus (17) and, although it remains controversial as to it's influence following long-term infection (43), it is now widely accepted that liver pathology associated with HBV infection is mediated primarily by the immune system following immune activation (42, 44, 45). In fact the outcome of HBV infection appears to depend heavily on the strength and quality of the HBV-specific immune response (42). Observations in humans have revealed that acute disease is associated with a strong and antigenically diverse immune response characterized by good cytotoxic T lymphocyte (CTL) activity.

In contrast, chronic disease is associated with weak and antigenically restricted immune response characterized by poor CTL activity (42, 46, 47). Unfortunately, effective animal models and *in vitro* systems for the study of HBV have been lacking due to the restricted host range of HBV to humans and great apes and the inability to infect cells in culture.

These barriers have been partially overcome through the creation of different HBV transgenic mouse models (48, 49). These models, some of which actually support complete viral replication and produce infectious virus, have yielded tremendous information with respect to HBV replication. With respect to immunopathology, the transgenic mouse models led to the direct observation for the first time that liver pathology associated with HBV infection was likely mediated in part by an HBV-specific cellular immune response. When major histocompatability complex (MHC) class I restricted CD8+ T cells, specific for HBsAg, were adoptively transferred into animals that were transgenic for HBsAg, the mice developed liver pathology reminiscent of acute viral hepatitis in humans (50). It appeared that, as with many other intracellular pathogens, direct CTL mediated killing of infected hepatocytes was a plausible mechanism by which HBV was eliminated from the body (51). However, based on the finite cytolytic potential of individual CD8+ T cells, it seems unlikely that a limited number of HBV-specific CD8+ T cells (approximately 0.03 for every hepatocyte) could be solely responsible for the pathology observed (48). Further investigation revealed that HBV-specific T cells initiated pathological changes, but subsequent

influxes of non-antigen-specific inflammatory cells were responsible for the majority of pathology observed (51, 52).

This, however, still did not complete the picture. The majority of adult infections with HBV are generally resolved with relatively mild clinical symptoms and little or no associated liver pathology (42). Furthermore, in the case of chronic infection, many carriers resolve their infection, either spontaneously or following interferon alpha (IFNα) therapy, with relatively mild self-limited exacerbation of their hepatitis (46). This is particularly significant given that the majority, if not all, hepatocytes are infected during chronic disease, therefore resolution of infection in such individuals, with limited associated pathology, suggests that the control of HBV infection is not solely mediated by destructive mechanisms.

New evidence from the transgenic mouse models suggested that HBV infection might also be controlled by non-cytopathic mechanisms. Working with the knowledge that hepatocyte gene expression was influenced by inflammatory cytokines, investigators hypothesized that these same cytokines may also have an influence on HBV gene expression in infected hepatocytes. Using mice that were transgenic for the 2.1 kb HBV mRNA species under the control of either HBV or cellular promoters, it was demonstrated for the first time *in vivo* that HBV gene expression was sensitive to inflammatory cytokines (53). Following intraperitoneal (IP) injection of a single sub-lethal dose of lipopolysaccharide (LPS), a selective 50-80% reduction in the 2.1 kb HBV mRNA content in the hepatocytes was observed. In an effort to identify the inflammatory cytokines responsible for the observed effect, recombinant forms of inflammatory cytokines

known to be induced by LPS were also evaluated. Similar effects on HBV mRNA content could be achieved with a single non-toxic dose of tumor necrosis factor alpha (TNFα) or high doses of IFNα or interleukin (IL) 6 (53).

Subsequent studies using recombinant IL-2 as a stimulator of cytokine production, supported a role for a TNFα dependent mechanism, but also identified a second apparently TNFα independent mechanism for the negative regulation of HBV gene expression. The TNFα independent mechanism was induced following the delivery of multiple doses of recombinant IFNα and IFNβ and was not blocked by prior administration of anti-TNFα antibodies, as was the case with IL-2 (54, 55). These studies also suggested for the first time that the negative regulatory effects induced by these cytokines were mediated by posttranscriptional mechanisms since the rate of transcription controlled by the HBV promoter, as assessed by nuclear run on assay, did not change (54).

The link between the negative regulation of HBV gene expression and an HBV-specific cellular immune response came when investigators demonstrated that the observed reduction in HBV gene expression induced by inflammatory cytokines could be achieved by adoptively transferring HBV-specific CD8+ T cells into the HBV transgenic mice (56-58). These studies revealed that in addition to the induction of an acute hepatitis-like phenomenon, there was a substantial negative regulation of HBV gene expression (>95% reduction in HBV mRNA content) throughout the livers of the transgenic mice as assessed by Northern blot analysis (56). It was observed that the negative regulatory affect asserted by HBV-specific CD8+ T cells was mediated by posttranscriptional destabilization of

both nuclear as well as cytoplasmic HBV mRNA, as assessed by nuclear run on assays (57). Further evaluation of the posttranscriptional mechanisms of HBV control supports the existence of two independent pathways of viral inactivation (58). The first involves the elimination of HBV nucleocapsid particles containing replicating genomes, and the second involves the destabilization of viral mRNA.

Evaluating the role of different inflammatory cytokines, investigators found that only pretreatment with anti-IFNγ or anti-TNFα was able to block the negative regulation of HBV gene expression (56) (58). Interestingly, antibody blocking of IFNγ or TNFα had no effect on the severity of the disease observed in these animal models (56). Conversely, HBV-specific CD8+ T cells from perforin and Fas ligand deficient mice had no effect on the negative regulation of HBV gene expression in transgenic mice (56). Furthermore, infection of HBV transgenic mice with non-HBV related murine viruses such as lymphocyte choriomeningitis virus, adenovirus, or cytomegalovirus, which all cause inflammatory responses in the liver, negatively regulated HBV transgene expression (59, 60).

Collectively, these observations suggested that the suppression of HBV replication occurs by two separate but closely associated mechanisms: (i) the recognition and killing of hepatocytes, but with very limited necroinflammatory effects, and (ii) the cytokine-mediated non-cytopathic control of HBV replication.

It follows then that, in addition to active elimination of infected cells by CTLs, a non-cytopathic control of viral gene expression may play an important role in the control of HBV infection in humans.

This theory was supported by a landmark study that revealed a bi-phasic immune response during a self-limiting acute HBV infection in chimpanzees (61). Following the kinetics of HBV replication and immune activation during acute HBV infection, investigators observed that virologic markers such as serum HBV DNA and intracellular replicative DNA intermediates appeared early and began to decrease by 10-12 weeks post infection. In contrast, an intra-hepatic T cell influx was barely detectable at 12-14 weeks, but was strongly detectable at 16-18 weeks post infection. A transient rise in serum alanine aminotransferase (sALT) levels and number of apoptotic cells was detectable, but only after the majority of virologic markers had been reduced to almost undetectable levels. Thus, these findings supported the theory that control of HBV infection occurs primarily by non-cytopathic mechanisms followed by limited cytopathic mechanisms that occurred at the peak of T cell influx.

The identification of IFN-γ and TNF-α as key cytokines involved in the non-cytopathic control of HBV infection suggests that this mechanism is related to the development of a T helper 1 (Th1) type immune response, which is likely regulated by antigen presenting cell (APC) production of IL-12. In fact, investigators have demonstrated that, in addition to CD8+ T cells, the non-cytopathic control of HBV replication can be achieved by adoptive transfer of HBV-specific CD4+ Th1 like T cells (62) or by the delivery of recombinant IL-12 alone (63).

The presence and strength of a Th1 type immune response to HBV is very important in determining the outcome of infection. In the case of the minority

(~5%) of otherwise healthy HBV vaccine recipients that do not respond to the current protein vaccine therapy, there appears to be a deficiency in their ability to generate HBV-specific CD4+ T cell responses (64-66). This could be indicative of what is occurring during chronic disease. Chronic carriers who eventually resolve HBV infection, either spontaneously or following immunotherapy, demonstrate an enhanced CTL response towards the virus as assessed by *in vitro* stimulation of HBV-specific CTLs (46). In fact, the level of CTL responsiveness in these individuals resembles the level of CTL responsiveness observed during acute infection (46). These observations support the hypothesis that chronic disease results from a failure to mount sufficient CD4+ and CD8+ T cell responses towards HBV. Appropriate stimulation of the immune system may be all that is required to promote an effective response.

1.6. Prophylaxis and therapy of HBV infection

As mentioned above, an effective prophylactic vaccine against HBV has been available since 1981. Initially made up of HBsAg purified from the plasma of chronically infected individuals, the vaccines are now comprised of recombinant HBsAg combined with aluminum hydroxide (alum), which acts as an adjuvant (67, 68). The vaccines are generally given as a series of 3 or 4 injections at 0, 1 and 6 months or 0, 1, 2, and 12 months to ensure the highest level of sero-protection among recipients. Sero-protection has been defined as anti-HBs antibody levels of >10 mIU/mL (69-72). These vaccines have proven to be effective at inducing protective immunity in 95% of healthy individuals. These vaccines were initially used widely only in perceived at-risk populations but

epidemiological data revealed no effect on the rate of appearance of new infections worldwide (14). Thus, based on preliminary data with population-wide vaccination programs supporting the reduction of neonatal transmission rates and the occurrence of HCC in adults, the World Health Organization (since 1991) now supports the adoption of HBV vaccines into the national vaccination programs of all countries around the world (14). However, despite these efforts, there still remains a large portion of the world's population that is chronically infected with HBV, and unfortunately, for these individuals, there still is no truly effective therapy to combat their infection (73-75).

IFNα has been the most widely used therapy to date, however it is only effective in about 35% of cases and is generally restricted to a subset of patients who present with favorable pre-therapy indicators that include, positive serum HBeAg, elevated sALT levels, and low HBV DNA levels (73-75). These indicators reveal the requirement of a degree of active hepatitis as a favorable pre-treatment presentation. IFN's are thought to function on two levels, both as an anti-viral as well as an immunomodulator for the amplification of existing immune responses (76). The currently recommended course of IFN therapy consists of either 5 million U daily or 10 million U three times a week given subcutaneously for 16 weeks, and is generally associated with a number of significant side effects such as, flu-like symptoms, mild bone marrow suppression, thyroid abnormalities and psychological depression (75).

A second category of therapeutic agents, the nucleoside and nucleotide analogues, have also been extensively studied (73-75). These compounds

compete with natural nucleosides and nucleotides during DNA synthesis. Their primary mode of action, based on their structure, is to destabilize DNA synthesis by interfering with the enzymatic function of the viral polymerase. Unfortunately, the first generation analogues that were investigated proved to be of little benefit and were associated with serious side effects (75). The second-generation analogues, however, appear to hold more promise, displaying more dramatic effects on HBV replication and better patient tolerance.

Lamivudine is the first of this new generation of analogues to be approved for the therapy of chronic HBV. Others in pre-clinical and clinical investigation include Famciclovir, Adefovir and Adefovir Dipivoxil, Ganciclovir, Labucavir, Entecavir, and Emtricitabine. Lamivudine, given at 100-300 mg daily, is well tolerated, dramatically reduces viral DNA in the serum to undetectable levels, and produced positive histological responses in a significant number of patients (52-56%) compared to placebo control (23-25%) (77). Finally, unlike with IFNα, successful treatment with Lamivudine is independent of pre-treatment variables (78, 79). The unfortunate drawback with Lamivudine, as well as other nucleoside analogues, is that relatively few individuals are actually cured of infection (73-75). Once therapy is stopped, viral DNA in the serum almost always quickly returns to pretreatment levels (77, 78, 80, 81), and although long term Lamivudine therapy is well tolerated it is also associated with the development of drug resistance (75).

A third, but less extensively studied category of chronic HBV therapy is therapeutic vaccines. The promise of therapeutic vaccines relies heavily on the

tight association between a strong anti-HBV immune response and control of HBV infection. Therapeutic vaccines attempt to stimulate the immune system in an effort to jump start an HBV-specific immune response that will go on to control viral infection. The most promising agent to be evaluated to date is Theradigm-HBV™ (73). It is a lipopeptide-based T cell vaccine that combines a CTL epitope isolated from HBcAg with a helper T epitope, initially from the influenza nucleoprotein and eventually from tetanus toxoid, and two palmitic acid molecules. In phase I clinical trial, Theradigm-HBV was well tolerated and did demonstrate the potential to induce an HBV-specific CTL response in healthy individuals (82). Unfortunately, further clinical study in chronically infected patients failed to induce measurable differences in CTL activity and revealed only minor effects on infection status (83).

1.7. CpG DNA

The identification of immunostimulatory DNA sequences present in bacterial DNA followed observations that bacterial DNA was capable of stimulating strong immune activation of vertebrate immune cells (84). These sequences have been defined as CpG motifs in which CpG dinucleotides are present within a particular base context (85). In prokaryotic organisms, CpG dinucleotides are present at the expected frequency of one in every sixteen pairs of bases along each single strand of DNA, whereas in vertebrate DNA, CpG dinucleotides, and as such CpG motifs, are suppressed to one third or one quarter of the expected frequency (86). In addition, within mammalian DNA the cytosine residues are highly methylated (86), which eliminates any immunostimulatory properties (85, 87).

These differences likely reflect an evolutionary divergence that has resulted in one of the many mechanisms by which vertebrates can recognize and respond to invading bacteria.

Both bacterial DNA and synthetic oligodeoxynucleotides (ODNs) synthesized using a nuclease resistant phosphorothioate backbone and containing CpG motifs, have been shown to induce or enhance the stimulation of a variety of immune cells. This stimulation includes: (i) the direct activation of murine and human B cells resulting in immunoglobulin production, IL-6 and IL-10 secretion, MHC class II and B-7 upregulation, and resistance to apoptosis (85, 88-91); (ii) the direct activation of macrophages and dendritic cells resulting in CD4 independent maturation and subsequent release of chemokines and pro-inflammatory cytokines including IL-12, upregulation of MHC class II, B-7s expression and CD40 expression (92-95); (iii) the activation of natural killer (NK) cells resulting in the rapid induction of IFNγ production and lytic capabilities (96-98). Although these affects appear to be largely mediated indirectly by the CpG DNA induced activation of APCs and subsequent production of IL-12, some observations have revealed that CpG DNA is capable of enhancing IFNγ production in response to IL-12 alone (99); and (iv) indirect influence on the activation of CD4+ and CD8+ T cells by the CpG DNA mediated activation of APCs and subsequent production of cytokines such as type I IFNs and IL-12, which lead to the development of a pro-Th1 immune environment (100-104). However, as with the NK cells, it appears that CpG can also have some direct ability to co-stimulate primary T cell activation (105).

The exact mechanism by which CpG DNA directly stimulates different cell populations still remains unclear. Results from early investigations supported a requirement for internalization of the CpG DNA (85). CpG DNA mediated stimulation could be blocked by inhibitors of endosomal acidification, suggesting that internalization involved the uptake of CpG DNA into endosomes, which subsequently underwent an acidification and maturation cycle (106, 107). However a CpG-specific receptor remained elusive. In the absence of an identifiable surface receptor it was thought that the CpG DNA might be translocated from the endosomes to the cytoplasm where it interacted with a putative CpG DNA-specific binding protein, which in turn mediated the intracellular signal cascade (108).

One of the earliest measurable events following stimulation with CpG DNA is the generation of reactive oxygen species (ROS) that is closely associated with endosomal acidification (109). It is thought that ROSs may have an important role in the regulation of the redox balance in leukocytes. This in turn influences the binding of transcription factors, such as AP-1, NF-κB, p53 and SP-1, during cellular activation (110-112).

How the signal-transduction cascade progressed from this point to the initiation of gene transcription was not known. At the transcriptional end of the cascade the two major transcription factors induced by CpG DNA are NF-κB and AP-1 (113). NF-κB is a key cellular transcription factor that is closely associated with the activation of immune cells of both the innate and acquired arms of the immune system (114). The activation of the NF-κB is dependent on the

phosphorylation, ubiquitination and subsequent degradation of the negative regulator of NF-κB, IκB. The signal transduction cascade induced by CpG DNA includes the activation of NF-κB (92), via the documented degradation of IκB (115). The CpG induced degradation of IκB following phosphorylation supports the upstream activation of intracellular kinases. One such kinase that has been proposed to mediate this process is DNA dependent protein kinase (DNA-PK) (116). Animals lacking the catalytic subunit of this enzyme fail to respond to CpG DNA (116). Therefore, it has been suggested that DNA-PK is involved in an upstream event that leads to the activation of IκB kinase (IKK) and subsequent degradation of IκB (116).

The second major group of signal transduction pathways that has been identified as being influenced by CpG DNA is the mitogen-activated protein kinase (MAPK) pathways (113). The MAPKs are a group of serine and threonine-specific kinases that play an important role in mediating responses to extracellular signals (117). The three best known pathways in this group, identified by the last kinase present in their respective cascades, are the extracellular-signal-regulated (ERK)/MAPK pathway, the c-Jun *N*-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway and the p38 pathway (117, 118). Each of these pathways has been reported to respond to CpG DNA and collectively lead to the generation and activation of the transcription factor AP-1 (113).

What has remained elusive, until recently, are the initiation events that lead to CpG DNA induced signal transduction cascades. Investigators have surmised that cellular activation by CpG DNA is similar to cellular activation following the

recognition of pathogen-associated molecular patterns (PAMP) present in other bacterial products such as LPS and bacterial lipoproteins. Using mice deficient in either the myeloid differentiation marker 88 (MyD88) or the tumor necrosis factor receptor-associated factor (TRAF) 6, investigators demonstrated that CpG DNA mediated activation was occurring via the Toll-like receptor (TLR)/IL-1 receptor (IL-1R) signaling pathway, much like with peptidoglycans and LPS-specific activation mediated by TLR-2 and TLR-4 respectively (119-121). The mammalian TLRs, so named for their striking homology to the toll receptors found in Drosophila (122), belong to the larger family of pattern recognition receptors (PRR) that play an important role in innate immune activation following the recognition of PAMPs (122). Both MyD88 and TRAF6 have been identified as adapter molecules in the TLR/IL-1R pathways (122) and the deficiency of MyD88 has a negative effect on LPS mediated activation (123). Based on the documented similarities in signal transduction between CpG DNA and other bacterial products containing PAMPs, it was predicted that CpG DNA-specific cell activation might be mediated in a similar fashion. However, knockout mice deficient in either TLR-2 or TLR-4 displayed normal responsiveness to CpG DNA-specific activation (119, 120). More recently it has been reported that TLR-9 deficient mice are completely unresponsive to CpG DNA, suggesting that TLR-9 may be the elusive CpG DNA-specific receptor, responsible for the upstream activation of the TLR/IL-1R signal transduction pathway (124).

Of particular interest from an immune modulation standpoint, is this ability of CpG DNA to promote Th1 biased immune activation. This is characterized by the

rapid production IFNγ and the biased production in some mice of the antibody isotype IgG2a (125), which is related to the induction of IL-12 production by antigen presenting cells (125, 126). It has been well established that IL-12 is a strong inducer of IFNγ production and is required for sustaining a Th1 biased immune response (127).

The immune stimulatory effects of synthetically derived ODNs containing CpG motifs makes them attractive candidates as immunological adjuvants, for the induction of humoral and cellular immune responses. In fact, many studies have already demonstrated that CpG ODNs are superior to other well-known adjuvants at inducing Th1 biased immune responses, including the gold standard, Complete Freunds' Adjuvant (CFA) (125, 128-134). Interestingly, the immunostimulatory properties traditionally associated with CFA, which results in a Th1 biased response, have since been attributed to the presence of CpG-containing mycobacterial DNA (125).

As with all agents with promise for human use, the potential for adverse effects is always a consideration. In the case of CpG DNA, the induction of inflammatory cytokines raises concerns over the risk of aberrant immune activation and the potential for the induction of septic shock (135, 136). However, neither has been reported at the dose levels used to achieve a desired immune stimulatory effect.

1.8. DNA Immunization

The use of plasmid DNA encoding antigenic proteins, known as DNA vaccines, for the induction of adaptive immune responses is a promising alternative to

traditional live attenuated, whole killed and subunit antigen-based vaccine strategies. Since their inception in the early 1990's (137-143), DNA vaccines have been evaluated in a variety of animal models and against numerous infectious agents and disease states (144-146), as well as in human clinical trials (147-152).

The exact mechanisms of action of DNA vaccines remain unclear (153). However, immunization with plasmid DNA is based on intracellular synthesis of the desired antigen(s) by the vaccine recipient. This leads to the development of antigen-specific immune responses. Following DNA-based immunization, immune activation can be detected for both CD4+ and CD8+ T lymphocytes via presentation of peptides in the context of the class II and class I MHC respectively, as well as for B cells via protein cross linking of antigen-specific surface immunoglobulin receptors.

After intra-muscular (IM) injection of a DNA vaccine, both myocytes and professional APCs may be transfected by the plasmid (137, 154). However, it is not clear the exact roles that each plays in the induction of immune responses. It has been shown that only APC such as dendritic cells can prime T-cell responses (154-158), and although studies on grafting stably transfected myoblasts showed that there is not an absolute requirement for direct transfection of APC (159, 160), it is not clear whether this would still be the case in the context of direct delivery of plasmid DNA. Furthermore, if not essential, it is not known what role direct transfection of APC might play.

Many investigators have demonstrated that IM delivery of plasmid DNA into mice induces strong and long-lasting immune responses comprising both antigen-specific antibodies and CTL (142,161-163). The finding that a single administration resulted in life-long immunity in mice (164) was initially thought to be due to the prolonged expression of antigen, as had been observed previously with certain reporter genes (165, 166). However, in the presence of a developing immune response, characterized by the generation of a strong MHC class I restricted CTL (161), it would seem likely that transfected myocytes and other cells would become potential targets for CTL mediated lysis. Indeed this seems to be the case when expression of a luciferase reporter gene is used as a marker of transfected myocyte integrity (167). Luciferase does not appear to be sufficiently immunogenic in the mouse to bring about rapid or complete destruction of transfected myocytes, hence strong expression can be detected for many months (165, 167). However, mice injected with a luciferase-encoding plasmid, together with an antigen-encoding plasmid expressing HBsAg or β-galactosidase (β-gal) lost the vast majority of luciferase-expressing myocytes, which presumably were co-transfected with antigen-expressing plasmids, within 20 days. This myocyte destruction was immune mediated since luciferase expression was lost in co-injected muscles of immune competent but not severe combined immunodeficient (SCID) mice (167). The myocyte destruction was not dependent on secretion of the synthesized antigen as demonstrated by the response towards β-gal, and was accompanied by an influx of CD8+ and CD4+ T cells, as assessed by immunohistochemical staining. Therefore, although the

period of antigen expression following DNA vaccination appears to be relatively short (approximately 2 weeks) it is nonetheless sufficient to induce long-lasting immune responses in mice without further boosting.

The potential advantages of DNA immunization over more traditional vaccine strategies are many (168). DNA vaccines generate both humoral and cellular immune responses, overcoming the barriers of protein sub-unit vaccines and more closely approaching natural immune responses to infection. However, unlike natural infection or live attenuated vaccines, there is no risk of associated pathology since genes associated with virulence can be omitted from the constructs. Similarly, unlike whole killed vaccines there are no concerns of toxicity related to pathogen inactivation. The chief safety concerns associated with DNA vaccination are the potential for the development of anti-DNA antibodies and the potential for plasmid integration that leads to an oncogenic event (168). However, neither has been reported. From a manufacturing standpoint, DNA vaccines represent a relatively low cost alternative, requiring simple storage, as compared to the more complicated process of generating and storing recombinant protein based vaccines.

As with the CpG DNA, of particular interest is the ability of DNA vaccines to induce an immune response that includes the development of cell mediated immunity (CMI). The presence of CTL activity as part of the generated immune response is indicative of a Th1 influence and is due, in part, to the presence of CpG motifs within the bacterial DNA plasmids (128, 169, 170). In fact, it has been demonstrated in mice that DNA vaccination plasmids can be optimized for

CpG DNA content, rendering them more efficient at inducing immune responses (171). The CMI promoting quality of DNA vaccines makes them an attractive candidate for use as prophylactic and therapeutic agents in situations where antigen-specific CMI responses are key to vaccine efficacy.

1.9. Rationale

HBV is a non-cytopathic enveloped DNA virus that can induce the development of necro-inflammatory liver disease. The severity of this disease and the ability of infected individuals to control viral infection are closely associated with the strength and quality of the HBV-specific immune response. The key factor in this immune response is the development of strong CMI. The difference between people who control infection and those who do not is clearly correlated with the strength of their HBV-specific CMI response.

Can a weak or non-response to HBV be overcome to eliminate chronic infection?

Observations in chronically infected individuals suggest that this is possible. The key factor being the augmentation of an HBV-specific CMI response.

Can this be achieved with immune modulating therapies?

To a limited extent it has been. There has been some success with the use of IFN therapy in a subgroup of chronically infected individuals. The scientific basis for this therapy is the augmentation of weak ongoing HBV-specific immune responses.

Can the CMI promoting properties of CpG ODNs and/or DNA vaccination be exploited to overcome week or non-responsiveness to HBV?

Preliminary evidence in an HBsAg transgenic mouse system similar to those described above, demonstrated that tolerance to HBsAg could be overcome by DNA vaccination (172). The breaking of tolerance involved the development of anti-HBs antibodies and, as with the transgenic systems described herein, the downregulation of HBsAg transgene expression. Subsequently it was confirmed that the effect was dependent on the T cell production of IFNγ (173).

More recently, the combination of CpG DNA in the form of ODNs wirh a current HBV vaccine demonstrated the capability of overcoming the hyporesponsiveness to this vaccine in orangutans (174). One of the characteristics of the current HBV vaccines is the existence of a subgroup of individuals that do not respond to vaccination. It has been suggested that this lack of response is also associated with a deficiency in HBsAg-specific CD4+ Th cell responses.

Therefore, evidence exists for the potential of CpG DNA and DNA vaccines in generating the quality of immune response that would have positive influence on the inadequate or non-responsiveness to HBV in individuals who remain chronically infected.

1.10. Hypothesis

Immunization with CpG ODN in combination with HBsAg, or with an HBsAg-expressing DNA vaccine, can induce HBsAg-specific cellular and humoral immune responses that are capable of overcoming non-responsiveness to HBV.

1.11. Specific Objectives

i. *To evaluate and compare the influence of CpG ODN and DNA vaccination on HBV-specific immune activation in HBsAg transgenic mice and congenic C57BL/6 control mice.*

ii. *To evaluate the relationship between immune activation and the longevity of antigen expression following DNA vaccination.*

iii. *To evaluate the influence of CpG ODN and DNA vaccination on HBV-specific immune activation in chimpanzees.*

2. Materials and Methods

2.1. Vaccine Reagents

Antigens

The protein antigen used for vaccination in the mouse studies consisted of recombinant HBV S2.S protein ayw subtype, produced in CHO cells (PMC, Val de Reuil, France and (175)). A 2 μg dose of recombinant S2.S_{ayw} protein was delivered in combination with a 100 μg dose of ODN.

The protein based vaccine used in the chimpanzee studies was Engerix B® (SmithKline Beecham, Rixensart, Belgium), a commercially available HBV vaccine consisting of 20 μg/mL of yeast derived recombinant HBV S_{ad} protein adsorbed to aluminum ions (Al^{3+}) provided by aluminum hydroxide (alum; 25 mg antigen/mg of Al^{3+}). A pediatric dose (10 μg of S_{ad}) of Engerix B was delivered either alone or in combination with a 1 mg dose of CpG ODN

DNA vectors

The DNA vaccines used in the mouse studies consisted of: (i) pCMV-S2.S plasmid (176) which express the S2.S (ayw) region of the HBV envelope gene under the control of the cytomegalovirus (CMV) immediate early gene promoter; (ii) An S_{ay}/luciferase colinear-expression vector (pCMV-S/CMV-luc) coding for both the HBV S_{ay} protein and luciferase, each under the control of its own CMV promoter that was generated by combining a 3.4 kb *SmaI-BamHI* fragment containing the luciferase reporter gene, under control of the immediate early

promoter of CMV that was isolated from the previously described pCMV-luc vector (177), gel purified, the 5' overhang filled in by DNA polymerase I large fragment, and cloned into the *StuI* site of the previously described pMAS-S vector (171), which contains the coding sequence for the HBV S_{ay} protein; and (iii) A pCMV-(-S)/CMV-luc control vector generated by first inserting 692 bp *EcoRV-PstI* fragment containing the HBV S_{ay} protein coding region that was isolated from the previously described pMAS-S vector (171) into a *PstI-SmaI* digested pMAS vector that did not contain the HBV S_{ay} protein coding region, which resulted in the insertion of the HBV S_{ay} protein coding region into the pMAS vector in the reverse (non-expressing) orientation and the creation of a pMAS-(-S) control vector. Subsequently, the 3.4 kb SmaI-BamHI CMV-luc fragment was cloned into pMAS-(-S) as described above generating the pCMV-(-S)/CMV-luc control plasmid.

The DNA vaccine used in the chimpanzee study, pCG-S, consisted of an HBV S_{ad} expressing DNA vector to which had been added 16 copies of the primate-specific CpG ODN 2006, which is a 24-mer with 3 CpG motifs, for a total of 64 additional CpG motifs (171).

All plasmid DNA was purified on Qiagen anion-exchange chromatography columns (Qiagen Inc., Valencia, CA, USA) as per the manufacturer's instructions and redissolved in 0.15 M saline for injection. The DNA was stored at -20°C until required.

ODN

The CpG ODNs used in the mouse studies consisted of: (i) CPG ODN (sequence1826: 5' TCCATGACGTTCCTGACGTT 3') (Coley Pharmaceutical Group, Inc., Wellesley, MA) and (ii) non-CpG ODN (sequence 1982: 5' TCCAGGACTTCTCTCAGGTT 3') (Coley Pharmaceutical Group Inc.). A 100 μg dose of ODN was delivered in combination with a 2 μg dose of recombinant $S2.S_{ayw}$.

The CpG ODN used in the chimpanzee study was the primate-specific CpG ODN 2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') (Coley Pharmaceutical Group Inc.). A 1 mg dose of ODN 2006 was delivered in combination with a pediatric dose of Engerix B (see above).

All ODNs were synthesised with a nuclease-resistant phosphorothioate backbone and suspended to 10 mg/ml in sterile endotoxin-free PBS (Sigma) and stored at 4°C until needed.

2.2. Mouse Studies

Animals

Immune competent mice were 6-12 week old female BALB/c or C57BL/6 mice (Charles River Laboratories, Wilmington, MA). Immune compromised mice were bred in the transgenic facility of the Loeb Health Research Institute, Ottawa. Both male and female mice were used at 6-12 weeks of age and the breeding pairs were originally obtained from: SCID C.B-17 SCID mice (Charles River), MHC

class I (C1D) and class II (C2D) deficient mice (Taconic, Germantown, NY) and previously described perforin knock-out (Pfn K/O) mice (178) (Sandoz Ltd. Zurich). The studies performed with these animals were presented to and accepted by the animal care of the LOEB Health Research Institute under the guidelines of the Canadian Council on Animal Care (CCAC).

Experimental groups

Influence of CpG ODN exposure on naïve splenocytes in-vitro

Naïve C57BL/6 mice (n=5) were sacrificed by cervical dislocation and their spleens harvested under sterile conditions. Single cell splenocyte cultures were established by the dissociation of spleens through metal screens. Splenocyte cultures were used to evaluate the effect of CpG ODN exposure on splenocyte activation as measured by cellular proliferation and cytokine production.

In-vivo immune response generated towards HBsAg following immunisation with pCMV-S2.S or recombinant S2.S_{ayw} combined with CpG ODN

C57BL/6 mice (n=10 per group) were immunised with either the pCMV-S2.S DNA vaccine or a protein based vaccine consisting of recombinant S2.S_{ayw} combined with either a CpG ODN or non-CpG ODN. The DNA vaccine was delivered as a single dose of 100 μg by bilateral IM injections into regenerating TA muscles as previously described (179). The DNA was dissolved in a total volume of 100 μl that was divided equally between the two injection sites.

The protein-based vaccine was delivered as a single dose, or three monthly doses, of 2 μg of recombinant S2.S_{ayw} combined with a 100μg dose of ODN. The

vaccine was administered by IM injection into non-regenerating TA muscles in a total volume of 100 μl, divided equally between the two legs, as previously described (131).

Blood was collected at 4 week intervals by retro-orbital bleed and assessed for anti-HBs antibody titers by endpoint dilution enzyme-linked immunosorbant assay (ELISA). Four weeks after the final vaccination, the animals were sacrificed by cervical dislocation, and their spleens harvested under sterile conditions. Single cell splenocyte cultures were established by the dissociation of spleens through metal screens and used to evaluate HBsAg protein-specific, cellular immune responses as assessed by CTL activity and cytokine production.

Influence of immune activation on antigen expression following DNA vaccination

Immuno-competent (BALB/c and C57BL/6) and immuno-compromised (SCID, C1D, C2D, and Pfn K/O) mice were injected IM bilaterally with a 50 μg dose of the pCMV-S/CMV-luc vector in the *tibialis anterior* (TA) muscle, as described previously (180). As a control for S_{ay} expression, separate groups of immuno-competent mice were injected bilaterally with a 50 μg dose of the control pCMV-(-S)/CMV-luc vector. Each experimental group consisted of 25 animals, of a particular strain, that were separated into subgroups of 5 animals. At 5 different time points following DNA injection (3, 7, 14, 21, 42 days) one subgroup of animals was sacrificed by cervical dislocation and their TA muscles harvested. The muscles were used to evaluate the influence of different immune effector mechanisms on antigen expression following DNA vaccination using an indirect assay for myocyte integrity, which was based on the sustained expression of

luciferase by intact myocytes following transfection with a mammalian expression vector encoding the luciferase gene.

Experimental procedures

In vitro splenocyte stimulation assay

Spleens from naïve C57BL/6 mice were recovered under sterile condition. Single cell suspensions were prepared in RPMI media (RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with L-glutamine (2mM), penicillin (100 U/mL), streptomycin (100ug/mL) and 2% (V/V) heat inactivated normal mouse serum (NMS) (Cedarlane, Hornby, Canada), and 0.02 mM β-2 mercaptoethanol (β2ME) (Sigma, St. Louis, MO)). The splenocytes were plated, $1x10^6$/mL for the proliferation assay and $5x10^6$/mL for the cytokine assays, at 100 µL/well in triplicate in 96-well round-bottom polystyrene plates (Becton Dickinson Labware, Franklin Lakes, NJ). The CpG ODN and the non-CpG ODN were suspended in RPMI media and plated at 100 µL/well to final concentrations of 10, 5, 2, 0.2 µg/mL for the proliferative assays, and 10, 3, 1, 0.3 µg/mL for the cytokine evaluation. For comparison, genomic *E. coli* DNA (Sigma), as a source of bacterial DNA, was suspended in complete RPMI 1640 and plated at 100 µL/well to final concentrations of 10 and 30 µg/mL. The lympho-proliferative assay was incubated for 96 hrs at 37°C and 5% CO_2 then pulsed overnight with tritiated thymidine (Amersham Pharmacia Biotech, Uppsala, Sweden) at 1 µCi/well. The cultures were harvested onto glass filters using an automated cell harvester (Skatron, Newmarket, Suffolk, UK) and the filters were allowed to dry

overnight. Thymidine incorporation was assessed by liquid scintillation counting using an automated beta counter (Beckman Coulter, Fullerton, CA). Proliferation was expressed as a stimulation index (SI), which was calculated as average counts per minute (cpm) of ODN driven proliferation over the average cpm of media background.

For cytokine evaluation, 4 additional plates were setup in parallel, and identical to the proliferation assay, and culture supernatants were harvested and stored at –80°C following 6, 24, 48, and 72 hrs of incubation at 37°C and 5% CO_2. Levels of TNFα, IL-6, IL-12, and IFNγ were determined using the murine OPTEIA ELISA sets (Pharmingen, Mississauga, Canada).

Assay for anti-HBs titers

Anti-HBs titers were determined in plasma recovered from mice, at various times after immunization, by ELISA (131). In brief, 96 well plates (Becton Dickinson) coated with plasma derived $HBsAg_{ay}$ (0.1 μg/well) (International Enzyme, Fallbrook, CA) were used to capture mouse plasma anti-HBs, which were then detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG3 (1/4000 in PBS/0.05% Tween 20/10% FBS, Southern Biotechnology Associates, Inc., Birmingham, AL), followed by *o*-phenylenediamine dihydrochloride solution (Sigma). Anti-HBs titers were defined as the highest plasma dilution that resulted in an absorbance (OD 450) two times greater that of non-immune plasma. Titers were expressed as the geometric

mean ± SD of values from individual animals in a treatment group, which were themselves the average of at least duplicate assays.

Assay for HBsAg-specific CTL activity

Single cell suspensions were prepared from spleens recovered from mice (n=5) 4 weeks following their final immunization. The responder spleen cells ($3x10^7$) were cocultured in a T-25 tissue culture flask (Becton Dickinson) with $1x10^6$ irradiated syngeneic transfected cells that expressed the small envelope protein (RBL5/S) (172), in 10 ml of RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2mM), penicillin (100 U/mL)/streptomycin (100ug/mL) (Life Technologies) and 0.02mM β2ME. After 6 days in culture, the cytolytic activity was determined by standard 4h ^{51}Cr release assay using U-bottomed 96-well polystyrene plates (Becton Dickinson) containing $5x10^3$ RBL5/S target cells/well. The target cells (10^6) were labeled with 200 μCi of $Na_2{}^{51}CrO$ (Amersham Pharmacia) for 1.5 to 2 h at 37°C and 5% CO_2 then washed three times. To control for non-S-specific lysis, cytolytic activity was also determined using syngeneic non-transfected cells (RBL5) (172). The effector cells were plated at E:T ratios of 100:1, 50:1, 25:1 and 6.25:1. Assay supernatants were harvested and counted using an automated gamma counter (Beckman Coulter). The percent-specific cytotoxicity was determined by the formula [experimental release – spontaneous release)/(maximum release – spontaneous release)] x 100. Maximum release was determined by complete cellular lysis using 2N HCl (Fisher Scientific, Nepean, ON). Spontaneous release was < 30% of maximum release unless otherwise indicated.

Assay for HBsAg-specific cytokine production

Single cell suspensions were prepared from spleens recovered from mice (n=5) 4 weeks following their final immunization. Spleen cells ($5x10^6$/mL) were suspended in RPMI 1640, supplemented with 2% (v/v) heat inactivated NMS, L-glutamine (2mM), penicillin (100 U/mL)/streptomycin (100ug/mL) (Life Technologies, Gaithersburg, MD), 0.02mM β2ME and plated in quadruplicate in 96 well plates at 100 µL ($5x10^5$cells)/well. The spleen cells were stimulated with media (negative control), plasma derived $HBsAg_{ay}$ (International Enzyme), at final concentrations of 3, 1, 0.3, and 0.1 µg/mL, and concanavalin A (Con A) (Sigma-Aldrich) at a final concentration of 10 µg/mL. Cultures were maintained at 37°C in 5% CO_2, and culture supernatants were harvested and stored at –80°C following 6, 24, 48 or 72 hrs of incubation. Levels of TNFα, IL-4, IL-5, and IFNγ were determined using the murine OPTEIA ELISA sets (Pharmingen, Mississauga, Canada).

Indirect assay of muscle fiber destruction

Following bilateral IM injection with either the pCMV-S/CMV-luc vector or the control pCMV-(-S)/CMV-luc in the TA muscles, groups of animals (n=5 per strain) were sacrificed by cervical dislocation (under anesthesia) at 3, 7, 14, 21 and 42 days post injection with plasmid DNA. The TA muscles were removed and luciferase activity was measured using the Promega luciferase assay system (Promega, Madison, WI, USA) as previously described (177). Results were expressed as group means ± SD of relative light units (RLU)/s/mg protein, with

protein contents being determined by the BioRad microassay procedure (BioRad, Mississauga, ON, Canada) (181).

Statistical analysis

The statistical significance of differences between group means, for antibody titers and IFNγ levels, of mice receiving different vaccine strategies was determined by one-factor analysis of variance followed by the Bonferroni test for multiple comparisons to evaluate differences between specific data sets within the particular analysis group. Statistical significance was defined as $p \leq 0.05$.

The statistical significance of differences between group mean luciferase activity in muscles taken from a given mouse strain injected with a given vector at various times after DNA injection was determined by one-factor analysis of variance followed by the Dunnett's multiple comparison test. Differences of values at other time points relative to the 3-day activity were considered significant with a $p \leq 0.05$.

2.3. Chimpanzee studies

Animals

Nine juvenile chimpanzees (*Pan Troglodytes*) weighing 24-33 kg, were used in this study. The animals were located at Bioqual (Rockville, MD), where they were housed individually in glass biocontainment suites and maintained according to American Association for Accreditation of Laboratory Animal Care guidelines. The studies performed with these animals were also presented to

and accepted by the animal care committees of the National Institutes of Health (Bethesda, MD) and of the LOEB Health Research Institute, which operates under the guidelines of the CCAC.

Experimental groups

Immunization protocol

Eight animals were vaccinated against HBV by IM injection to the right quadricep muscle at 0 or 4 weeks with either: (i) pediatric dose of Engerix B alone (n=2) delivered by hypodermic syringe with a 23 gauge needle; (ii) pediatric dose of Engerix B combined with a 1 mg dose of CpG ODN (n=2) delivered by hypodermic syringe with a 23 gauge needle; (iii) a 2 mg dose of pCG-S (n=2) delivered bilaterally using the Biojecter needle less injection system (Bioject, Portland, OR); or (iv) a 2 mg dose of pCG-S at week 0 followed by a pediatric dose of Engerix B combined with a 1 mg dose of CpG ODN at week 4 (n=2). As a positive control for productive infection following challenge at 8 weeks, a ninth animal received an injection of normal saline (Sigma) at 0 and 4 weeks.

Throughout the immunization protocol the animals were evaluated for the induction of HBsAg-specific immune responses as assessed by antibody production, antigen-specific T cell proliferation, cytokine production, antigen-specific CTL activity.

HBV challenge protocols

At 8 weeks after prime each animal was challenged with $10^{3.5}$ chimp infectious dose 50 (CID_{50}) of HBV strain MS-2 (National institute of Health, Bethesda, MD) IV as a single bolus. Following challenge, the animals were evaluated for the development of a productive HBV infection, through detection of both HBsAg and HBeAg in the circulation.

Experimental procedures

Blood collection

The animals were bled prior to and on a weekly basis following vaccination. Venous blood was collected into heparinized vacutainers (Becton Dickenson) by venous puncture. Prior to lymphocyte isolation, the whole blood was allowed to separate for the collection of plasma.

Isolation of peripheral blood mononuclear cell (PBMC)

PBMCs from each animal were separated from whole blood by density gradient centrifugation on Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). The PBMCs were washed twice with PBS (Life Technologies) and used for culture directly or cryogenically preserved in media containing 50% heat inactivated FBS (Life Technologies), 40% RPMI 1640 (Life Technologies) supplemented with L-glutamine (2mM), penicillin (100 U/mL), streptomycin (100 ug/mL), and 10% (v/v) FBS (complete RPMI), and 10% di-methyl sulfoxide (Fisher Scientific).

Generation of Epstein Barr virus (EBV)-transformed cell lines

EBV transformed B cell lines were established from PBMCs recovered from each chimpanzee prior to immunization. PBMCs were cultured in supernatant collected from the EBV-producing marmoset cell line B95-8 (ATCC# CRL-1612), in complete RPMI supplemented with cyclosporin A (1 μg/mL) (Sigma-Aldrich). The cells were maintained in complete RPMI 1640.

Assay for anti-HBs antibody titers and Ig isotypes

Anti-HBs levels in plasma recovered from each animal were assessed using the Monolisa 3.0 anti-HBs ELISA kit (Sanofi Diagnostics Pasteur, Montreal CANADA). Anti-HBs levels were expressed in mIU/mL by comparison with human derived standards defined by the World Health Organization (Monolisa Anti-HBs Standards, Sanofi Diagnostics Pasteur). Seroprotection was defined as ≥10 mIU/mL.

The relative levels of different anti-HBs antibody isotypes were determined by endpoint dilution ELISA (182). In brief, 96 well plates (Corning) coated (1 μg/mL) with plasma derived $HBsAg_{ad}$ (International Enzyme) were used to capture anti-HBs from chimp plasma. The anti-HBs antibodies were detected with HRP-conjugated mouse anti-human IgG, IgG1, IgG2, IgG3, or IgG4 (1/2000 in PBS/0.05% Tween 20,10% FBS; Southern Biotechnology Associates, Inc.), followed by *o*-phenylenediamine dihydrochloride solution (Sigma). Anti-HBs titers were defined as the highest plasma dilution that resulted in an absorbance (OD 450) two times greater that of non-immune plasma.

Assays for HBsAg-specific T cell proliferation and cytokine production

HBsAg-specific T cell proliferation was assessed using a standard ^{3}H-thymidine incorporation assay. PBMCs were suspended in RPMI 1640, supplemented with 10% (v/v) heat inactivated human AB serum ($1x10^6$cells/mL) and plated in quadruplicate in 96 well plates at 100 µL ($1x10^5$cells)/well. The stimulants for the assay were: media (negative control), plasma purified $HBsAg_{ad}$ (International Enzyme) at final concentrations of 5, 2.5, and 1.25 µg/mL, and phytohemagglutinin (PHA) (Sigma) at a final concentration of 10 µg/mL. Cultures were maintained for 6 days at 37°C in 5% CO_2 then pulsed with ^{3}H-thymidine (Amersham Pharmacia) at 1 µCi/well and incubated for 18 hrs. The cultures were harvested onto glass filters using an automated cell harvester (Skatron, Newmarket, Suffolk, UK). The filters were allowed to dry overnight and thymidine incorporation assessed by liquid scintillation counting using an automated beta counter (Beckman Coulter). T cell proliferation was expressed as an SI, which was calculated as average cpm of antigen driven proliferation/average cpm of media background.

In parallel to the proliferation assay, PBMCs ($2x10^6$cells/mL) from each animal were cultured in sextuplets as described above in each of four 96 well plates for the evaluation of HBsAg-specific cytokine production. Culture supernatants were harvested following 6, 24, 28, or 72 hrs of incubation and stored at –80°C. Cytokine levels were assessed using Pharmingin's OptEIA ELISA sets (Pharmingen) for human TNFα, IL-4, IL-5, and IFNγ.

Preparation of stimulator and target cell lines for vaccinia based CTL assay

The HBsAg expressing cells used as stimulators and targets for the vaccinia virus based CTL assay were generated by infecting autologous EBV transformed B cells from each animal with recombinant vaccinia virus coding for the HBV S protein (rVV-S) at a multiplicity of infection (MOI) of 10. Infections were performed in a volume of 1.5 to 2 mL of RPMI supplemented with 2.5% FBS for 2 hrs at 37°C and 5% CO_2. Cultures were brought to 10 mL with complete RPMI and incubated over-night at 37°C in 5% CO_2.

Vaccinia virus based assay for HBsAg-specific CTL activity

Freshly isolated PBMCs were suspended in complete RPMI supplemented with recombinant human IL-2 (20 U/mL) (Roche Diagnostics, Laval, Que.) and plated in 24-well polystyrene plates (Becton Dickinson Labware, Franklin Lakes, NJ) at $4x10^6$ cells per well. HBV S expressing autologous EBV transformed B cells, irradiated at 5000 rads, treated with psoralen (Sigma) (10 μg/mL) and UV light for 10-15 minutes, were added to the PBMC cultures at $4x10^5$ cells per well in complete RPMI supplemented with recombinant human IL-2 (20 U/mL). The co-cultures were incubated for 7 to 8 days at 37°C in 5% CO_2 and fed at 3 day intervals with complete RPMI supplemented with recombinant human IL-2 (20 U/mL). On Day 7 or 8, cytolytic activity was determined using a standard 4h ^{51}Cr release assay using U-bottomed 96-well polystyrene plates (Becton Dickinson) containing 5000-target cells/well. As a control for non-S-specific lysis, autologous EBV transformed B cells were infected with wildtype vaccinia virus only (VV-WT). Target cells were labeled with 200 μCi of $Na_2{}^{51}CrO$ (Amersham

Pharmacia) for 1.5 to 2 h at 37°C in 5% CO_2 then washed three times. The effector cells, depending on cell number, were plated at E:T ratios of 100:1, 50:1, 25:1 and 6.25:1, or 50:1, 25:1, 12.5:1, and 6.25:1. Assay supernatants were harvested and counted using an automated gamma counter (Beckman Coulter). The percent-specific cytotoxicity was determined by the formula [experimental release – spontaneous release)/(maximum release – spontaneous release)] x 100. Maximum release was determined with 2N HCl (Fisher Scientific,). Spontaneous release was < 30% of maximum release unless otherwise indicated.

Peptide based assay for HBsAg-specific CTL activity

As an alternative to using recombinant vaccinia virus, CTL activity was also assessed using synthetic HBV peptides. A panel of 20 synthetic 20 amino acid long overlapping peptides (provided courtesy of SmithKline Beecham) that span the HBV S protein were separated into two pools of 10 peptides each (Pool 1 and Pool 2). Each pool was suspended to a final concentration of 10 μg/mL/peptide in RPMI for a total peptide concentration of 100 μg /mL. Cryogenically preserved PBMCs were rapidly thawed, washed once, and plated at 4 to $5x10^6$ cells/mL/well in 24-well polystyrene plates in complete RPMI supplemented with recombinant human IL-2 (20 U/mL), and pooled synthetic HBV S peptides at 5 μg/mL. The cultures were incubated for 14 days at 37°C in 5% CO_2, fed at 3 day intervals with complete RPMI supplemented with recombinant human IL-2 (20 U/mL), and on day 7 the cultures were re-stimulated with synthetic HBV peptide at 5 μg/mL. On day 14 the cytolytic activity was determined using a standard 4h

^{51}Cr release assay as described above. The targets for this assay were generated by incubating $1x10^6$ autologous EBV transformed B cells in 100 µL of peptide pool for 1 hr at 37°C and 5% CO_2. The cells were suspended at $2x10^6$ cells/mL and incubated overnight at 37°C in 5% CO_2. As a control for non-specific lysis, non-peptide pulsed EBV transformed B cells were also used as targets. The percent-specific cytotoxicity was determined as above.

Assay for circulating HBsAg

The presence or absence of circulating HBsAg in plasma recovered from each animal was assessed using the AUSZYME MONOCLONAL enzyme-linked immunoassay (EIA) kit (ABBOTT Laboratories, Abbott Park. IL) as per the manufacturers directions.

Assay for circulating HBeAg

The presence or absence of circulating HBeAg in plasma recovered from each animal was assessed using the ABBOTT HBe (rDNA) EIA kit (ABBOTT Laboratories) as per the manufacturer's directions.

3. Results

3.1. Influence of CpG ODN on immune activation in C57BL/6 mice.

The influence of CpG ODN on innate immunity and of S2.S + CpG ODN on antigen-specific immune responses in C57BL/6 mice were compared to those induced with an S2.S-expressing DNA vaccine. This work was done to determine the potential for non-cytopathic control of transgene expression in HBsAg-transgenic (Tg) mice that are congenic to C57BL/6 mice, using the same vaccines. Such Tg mice are a model for HBV chronic carriers who do not have active hepatitis, such as frequently happens after neonatal infection. It had previously been shown that an HBV-specific DNA vaccine could bring about non-cytopathic control of HBV gene expression in these Tg mice (172). The DNA vaccine served as a positive control in the present study, which was carried out as part of a collaborative project to see if S2.S + CpG ODN could have similar effects on the Tg mice. The evaluation of transgene expression was carried out by our collaborators at the Pasteur Institute (Paris) while the evaluation of antigen-specific immune responses was carried out by myself in Ottawa.

CpG ODN effectively induces a non-antigen-specific proliferation and cytokine production by naïve C57BL/6 splenocytes in vitro.

To evaluate the non-specific effects of CpG ODN on immune cells derived from non-immunized C57BL/6 mice, pooled splenocytes from 5 mice were cultured *in vitro* either alone, or with CpG ODN, non-CpG ODN or *E. coli* DNA (as a natural source of CpG motifs).

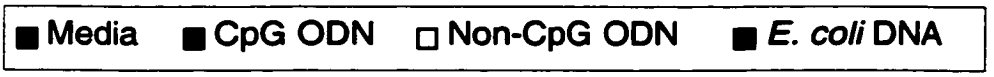
The data clearly demonstrated that CpG ODN (28,465 CPMs) was superior to media (80 CMPs), the non-CpG ODN (203 CPMs), or *E. coli* DNA (90 CPMs) at inducing a non-specific splenocyte proliferative response (Fig. 1.).

With respect to the induction of cytokine production, the CpG ODN was also superior to the non-CpG ODN and *E coli* DNA for TNF-α and IL-6 and to the non-CpG ODN for IL-12 and IFN-γ (Fig. 2). Comparable amounts of IFN-γ and IL-12 were produced by splenocytes after stimulation with either CpG ODN or *E. coli* DNA.

S2.S + CpG ODN effectively induced a strong anti-HBsAg humoral response but no detectable HBsAg-specific CTL response in normal C57BL/6 mice.

To evaluate and compare the effect of protein + CpG ODN and DNA vaccines on the generation of antigen-specific humoral and cellular responses, C57BL/6 mice were immunised IM three times at monthly intervals with recombinant S2.S combined with CpG or non-CpG ODN, or once with pCMV-S2.S plasmid DNA. Anti-HBs titers were determined by endpoint ELISA using plasma recovered from mice 4 weeks after their final immunization. The combination of S2.S with CpG ODN induced a significantly higher anti-HBs titer (mean±SEM; 739,663 ± 147,746) than the non-CpG ODN (214,208 ± 33,348) or pCMVS2.S (4,828 ± 890) ($p<0.001$) (Fig.3). There was no significant difference between the anti-HBs titers generated by pCMVS2.S (4,828 ± 890) and the S2.S with the non-CpG ODN (214,208 ± 33,348) ($p>0.5$).

Figure 1. Cellular proliferation following *in vitro* stimulation of naïve C57BL/6 splenocytes with CpG ODN. Splenocytes from 5 naïve mice were pooled and cultured in media alone with CpG or Non-CpG ODN at 5 μg/mL or *E.coli* DNA at 30 μg/mL, and proliferation was assessed by thymidine incorporation following 96 hrs. The data are representative of duplicate experiments.



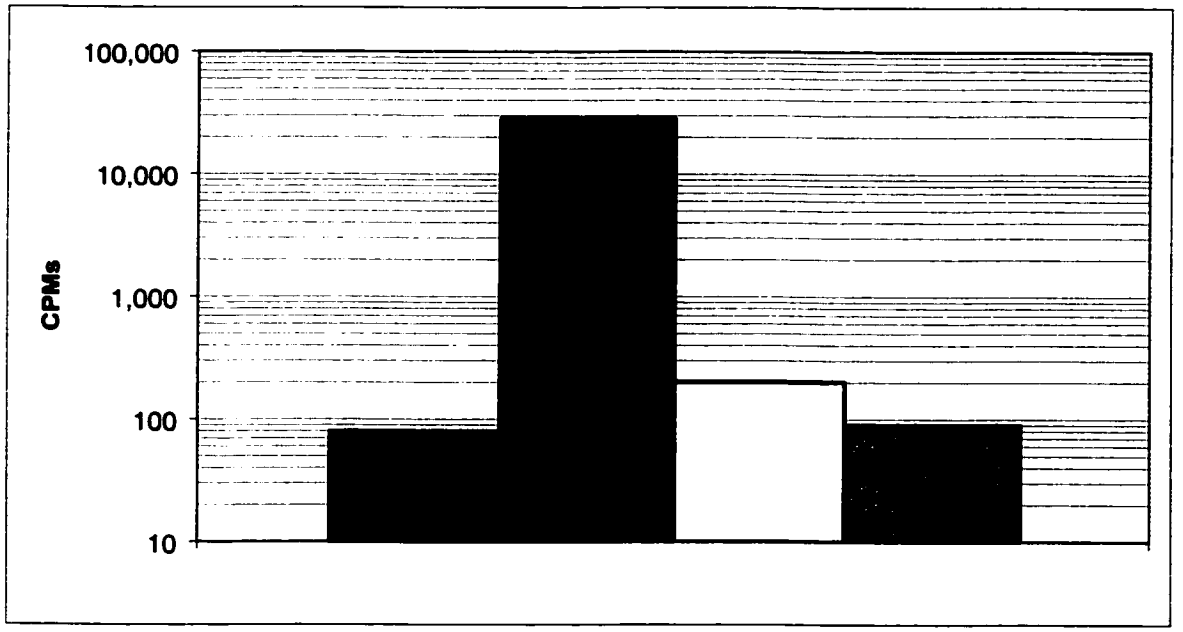
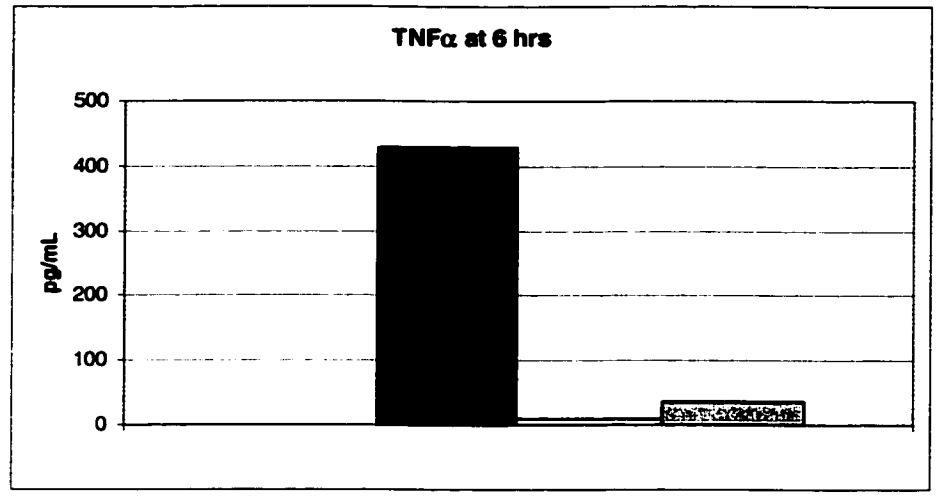
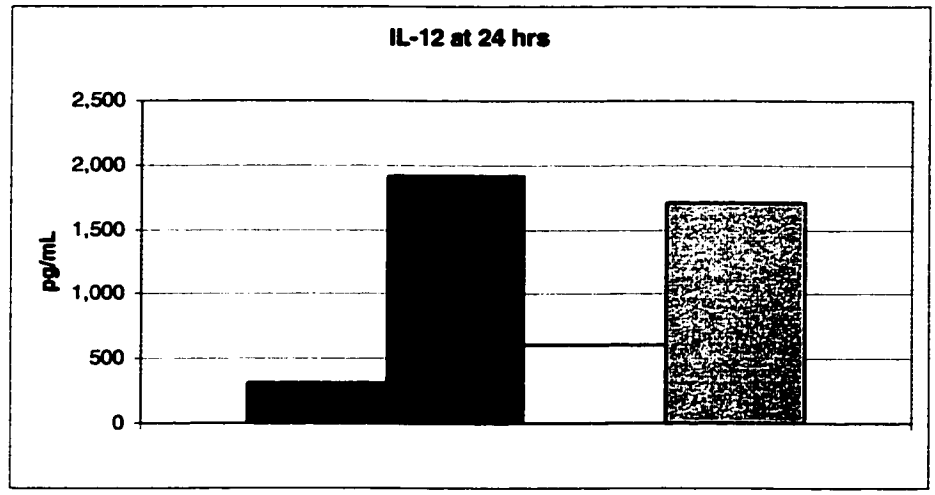
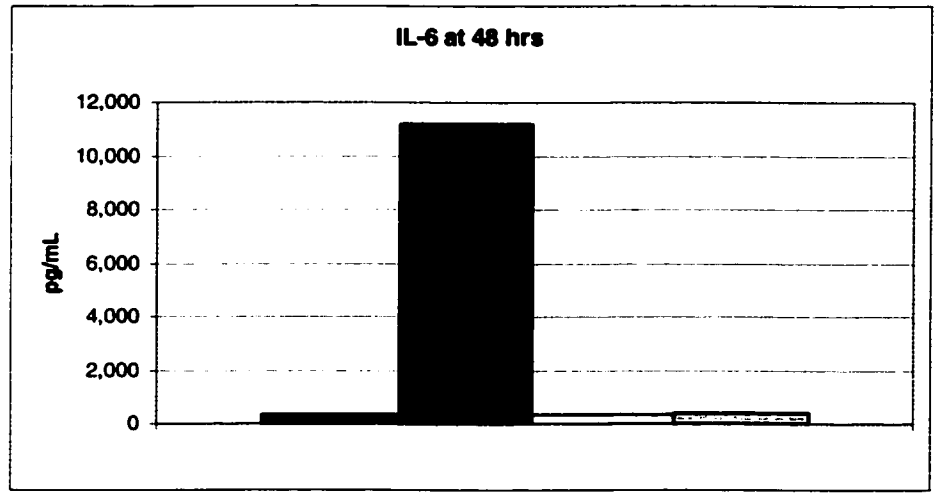

100,000
10,000
1,000
100
10
CPMs

Figure 2, Cytokine production following *in vitro* stimulation of naïve C57BL/6 splenocytes with CpG ODN. The production of TNFα, IL-12, IL-6, and IFNγ, by pooled splenocytes from 5 naïve mice cultured with CpG or Non-CpG ODN at 3 μg/mL or *E.coli* DNA at 30 μg/mL was measured in culture supernatants by commercial ELISA. The data are representative of duplicate experiments.


TNFα at 6 hrs
500
400
300
200
100
0
pg/mL


IL-12 at 24 hrs
2,500
2,000
1,500
1,000
500
0
pg/mL


IL-6 at 48 hrs
12,000
10,000
8,000
6,000
4,000
2,000
0
pg/mL

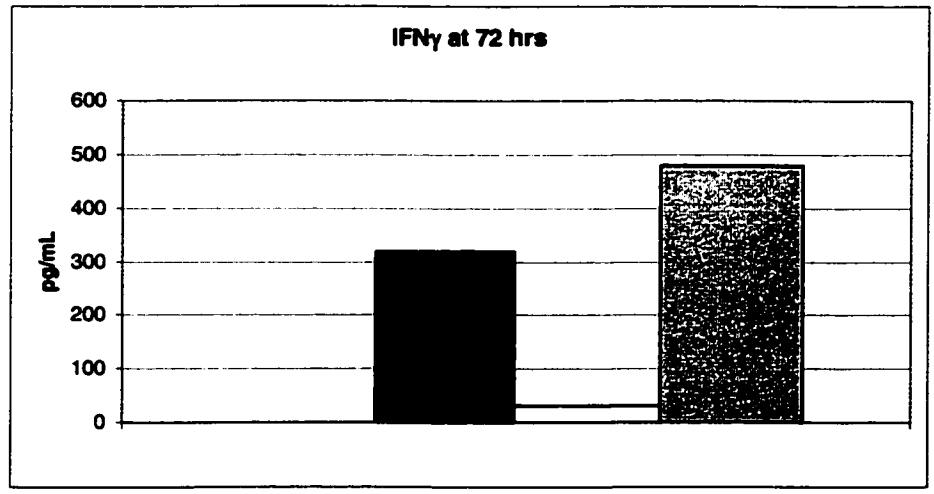
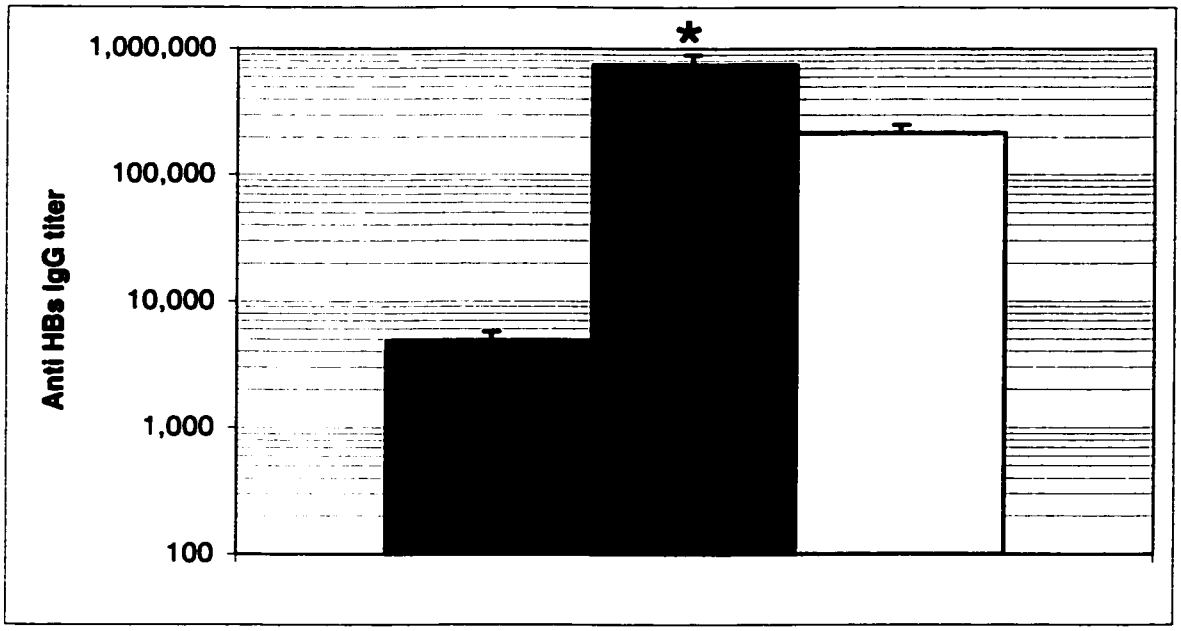

IFNγ at 72 hrs
600
500
400
300
200
100
0
pg/mL

Figure 3. Anti-HBsAg humoral response in C57BL/6 mice following immunization. Mice were immunized IM once with 100 μg of pCMVS2.S, or three times at 0, 4, and 8 weeks with 2 μg of recombinant S2.S in the presence of 100 μg of CpG ODN or non-CpG ODN. Anti-HBs antibody titers were determined from plasma recovered from mice at 4 weeks after their final immunization, by ELISA. Titers are expressed as the geometric mean ± SEM with *=p<0.001 when compared to pCMVS2.S and S2.S+non-CpG ODN (pCMVS2.S n=7, S2.S+ ODN n=10, S2.S+ non-CpG ODN n=10)


Anti HBs IgG titer
1,000,000
100,000
10,000
1,000
100
*

With respect to antibody isotypes, IgG2a levels were not determined due to the absence of this gene in C57BL/6 mice (183-185). There was a higher level of IgG2b compared to IgG1 in mice that received CpG ODN (data not shown), however this has not been confirmed to be indicative of a Th1 biased response in C57BL/6 mice.

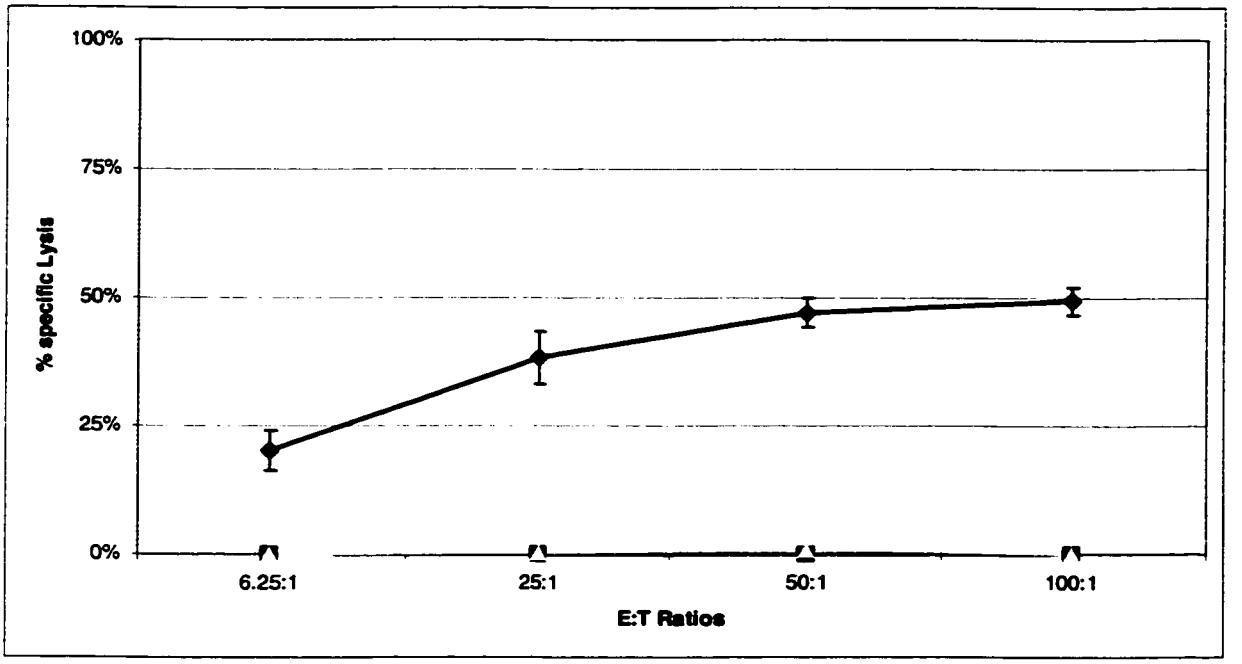
With respect to cellular immune responses, the combination of S2.S with CpG ODN did not induce a detectable HBsAg-specific CTL response in C57BL/6 mice after a single (Fig 4A) or triple (Fig 4B) immunization in contrast to a single (Fig 4A) pCMVS2.S immunization, which did induce a detectable HBsAg-specific CTL response.

Immunization with pCMVS2.S leads to more HBsAg-specific IFNγ production than immunization with S2.S + CpG ODN in C57BL/6 mice.

Based on the importance of IFNγ in the generation of CMI (186) and in the non-cytopathic control of HBV gene expression (56, 58, 173) and to evaluate the cytokine secretion profile of HBsAg specific T cells, the amount of HBsAg-specific TNFα, IL-4, IL-5, and IFNγ production by splenocytes *in vitro* was determined following each vaccination strategy (Fig. 5). A single immunization of C57BL/6 mice with pCMVS2.S led to a significantly higher level (mean±SD; 273 ±300 pg/mL) of HBsAg-specific IFNγ production than a single immunization with S2.S + CpG (97 ±52 pg/mL)($p<0.01$). No detectable TNFα, IL-4, or IL-5 was observed for any of the vaccine strategies.

Figure 4. HBsAg-specific CTL response in C57BL/6 mice following immunization. Mice were immunized IM once with 100 μg of pCMVS2.S (A), or once (A) or three (B) times at 0, 4, 8 weeks with 2 μg of recombinant S2.S in the presence of 100 μg of CpG ODN or non-CpG ODN. Spleens were harvested 4 weeks following the final immunization, restimulated *in vitro* for 6 days with transfected H-2^b restricted stimulators expressing HBsAg. A standard chromium release assay was performed on Day 6 using transfected H-2^b restricted targets expressing HBsAg. The data is represented as mean-specific lysis ± SD with n=5.

A


100%
75%
50%
25%
0%
% specific Lysis
6.25:1
25:1
50:1
100:1
E:T Ratios

B

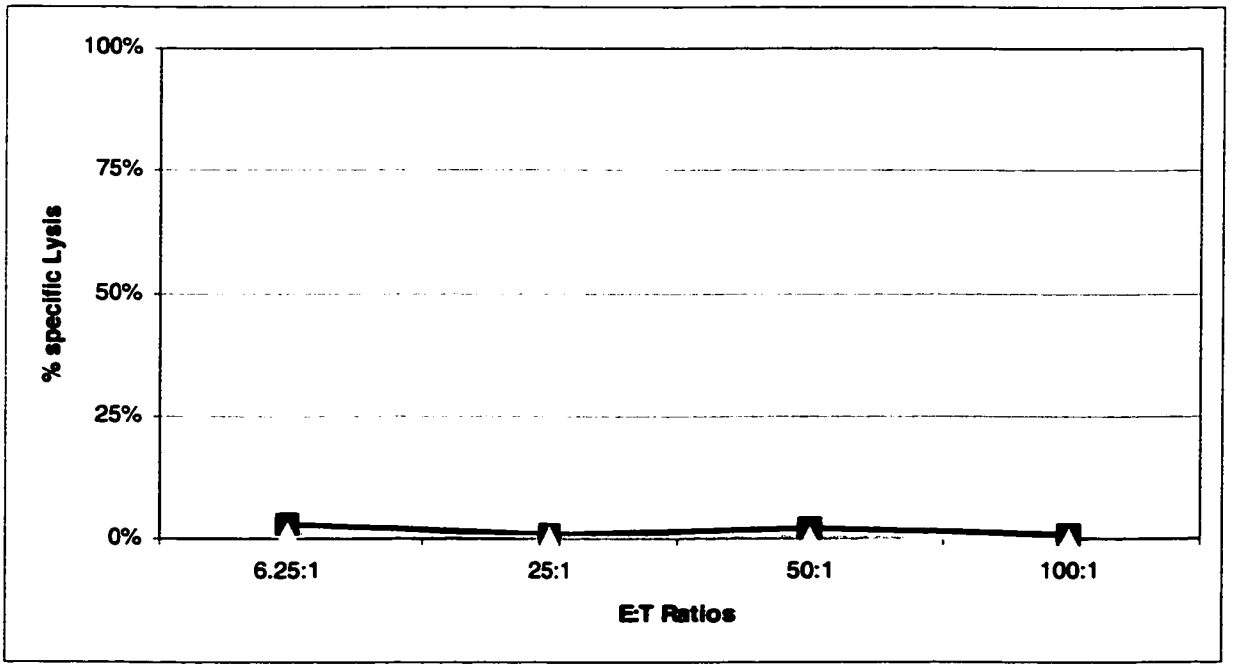
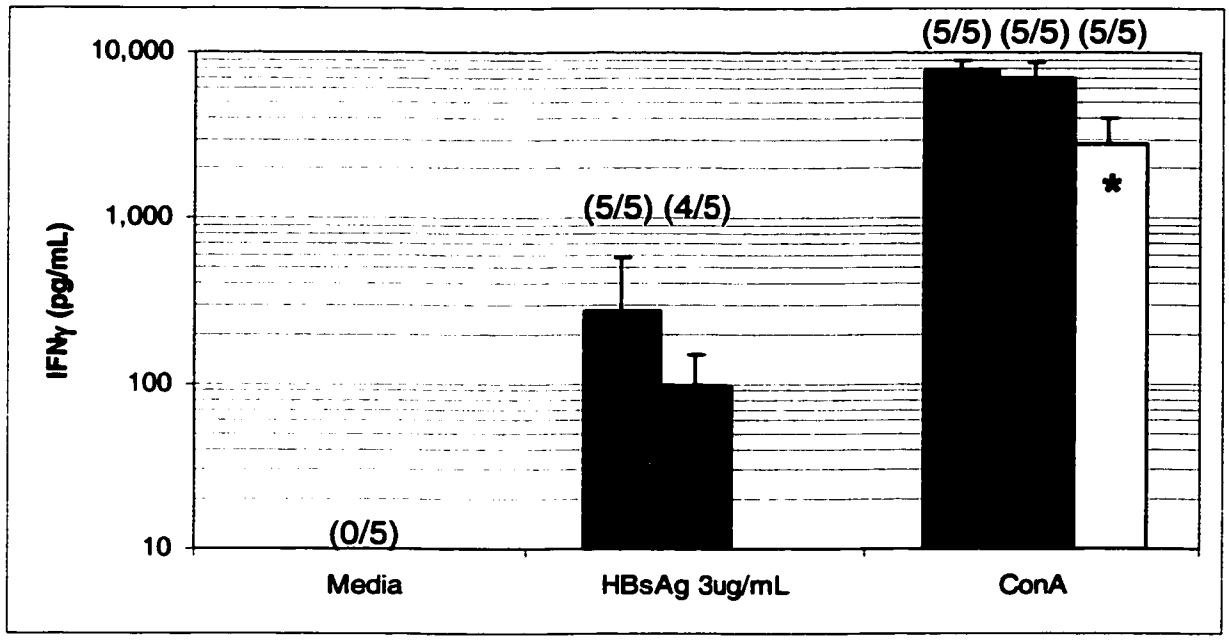

100%
75%
50%
25%
0%
% specific Lysis
6.25:1
25:1
50:1
100:1
E:T Ratios

Figure 5. I*n vitro* HBsAg-specific induction of IFNγ production by C57BL/6 splenocytes following immunization. Mice were immunized IM once with 100 μg of pCMVS2.S or 2 μg of recombinant S2.S in the presence of 100 μg of CpG ODN or non-CpG ODN. Splenocyte were cultured at 4 weeks post injection either alone, with HBsAg at 3 μg/mL or Con A at 10 μg/mL for 72 hrs. IFNγ production was evaluated in the supernatants using a commercial ELISA. The data is represented as means ± SD of responding animals. (#/#) represents the number of responders of total number of animals in group as determined by a cut off of 40 pg/mL. *=p<0.01 when compared to media control. **=p<0.01 when compared to S2S +CpG ODN following restimulation with HBsAg at 3 μg/mL.


10,000
1,000
100
10
IFNγ (pg/mL)
(5/5) (5/5) (5/5)
(5/5) (4/5)
(0/5)
*
Media
HBsAg 3ug/mL
ConA

3.2. Relation between immune activation and the longevity of antigen expression following DNA vaccination.

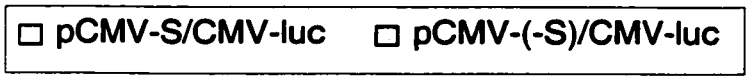
To better understand why DNA vaccination resulted in a stronger production of IFNγ than S2.S+CpG in the mice, the relation between immune activation and the longevity of antigen expression following IM delivery of a DNA vaccine was evaluated.

DNA vaccination induced an antigen-specific and immune mediated destruction of transfected myocytes following intramuscular injection.

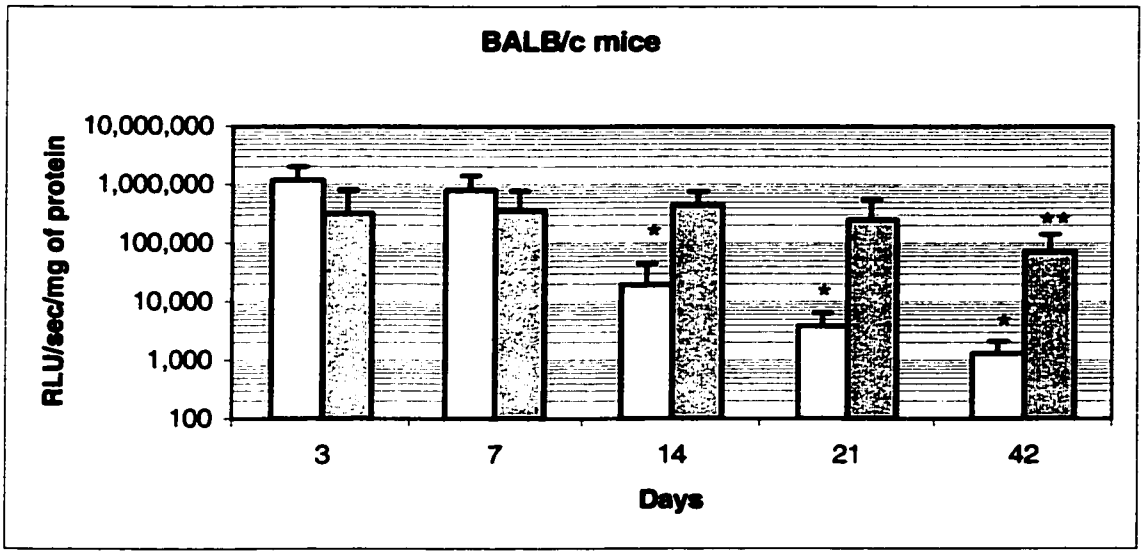
To determine the role of antigen on the destruction of muscle cells following DNA immunization, 50μg of the pCMV-S/CMV-luc vector coding for both HBV S and luciferase was delivered to the TA muscles of normal BALB/c (H-2^d) and C57BL/6 mice (H-2^b). Luciferase expression was assessed at different time points following injection and relative to the basel level found at 3 days, and found to be significantly decreased by 14 days in the BALB/c mice (Fig 6 A) and by 21 days in C57BL/6 mice (Fig 6 B). Levels continued to decline such that by 42 days, activity in both strains was only 0.1 % and 1% respectively of day 3 basal levels. In contrast, when 50μg of the pCMV-(-S)/CMV-luc control vector, which does not express the HBV S protein, was delivered to the TA muscles of normal BALB/c and C57BL/6 mice, a significant decrease in luciferase expression was only observed at 42 days, with the level of activity being 22% and 2% respectively of that detected at 3 days (Fig 6 A and 6 B).

To evaluate the role of antigen-specific immune responses in mediating the destruction of muscle cells following DNA immunization, 50μg of the S/luciferase

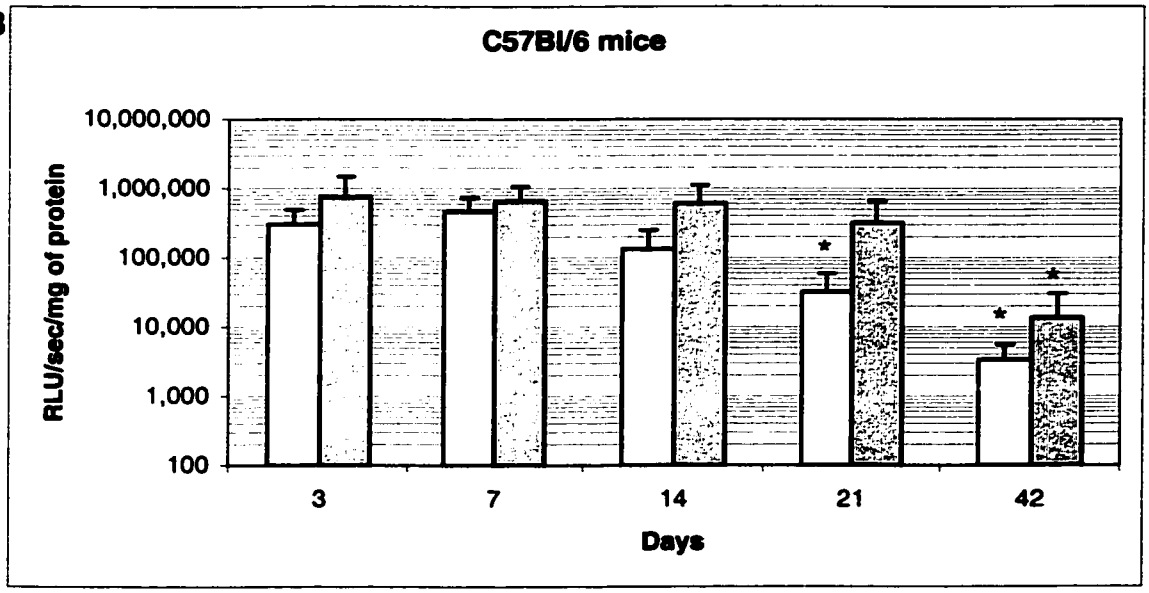
Figure 6. Myocyte destruction was associated with the expression of HBsAg. 50μg of the HBsAg/luciferase co-linear expression vector or control vector were delivered IM to the TA muscle of normal BALB/c (A) and C57BL/6 (B) mice. The TA muscles (n=10) were removed at different time points post injection and the level of luciferase expression assessed by commercial assay. The data is represented as means ± SD with **=$p<0.05$ and *=$p<0.01$ when compared to the day 3 values.



A


BALB/c mice
RLU/sec/mg of protein
10,000,000
1,000,000
100,000
10,000
1,000
100
3
7
14
21
42
Days

B


C57Bl/6 mice
RLU/sec/mg of protein
10,000,000
1,000,000
100,000
10,000
1,000
100
3
7
14
21
42
Days

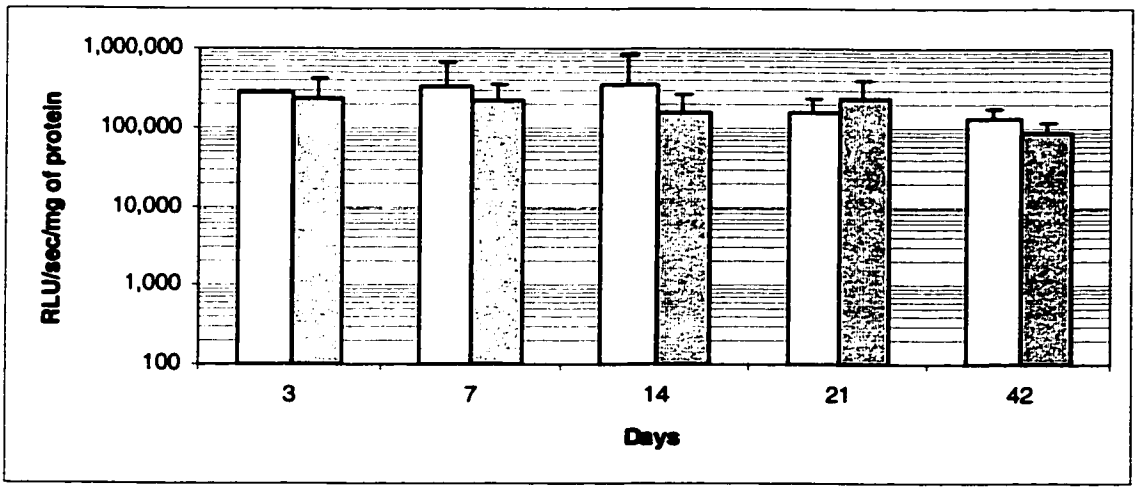
colinear-expression vector was delivered to the TA muscles of C.B-17 SCID mice (H-2^d). C.B-17 SCID mice fail to mount a functional adaptive immune response to specific antigen by virtue of a complete lack of B- and T-cells. Unlike the normal immune competent mice (Fig 6 A and B), there was no significant decrease in luciferase expression over 42 days in the injected muscles of the SCID mice (Fig 7).

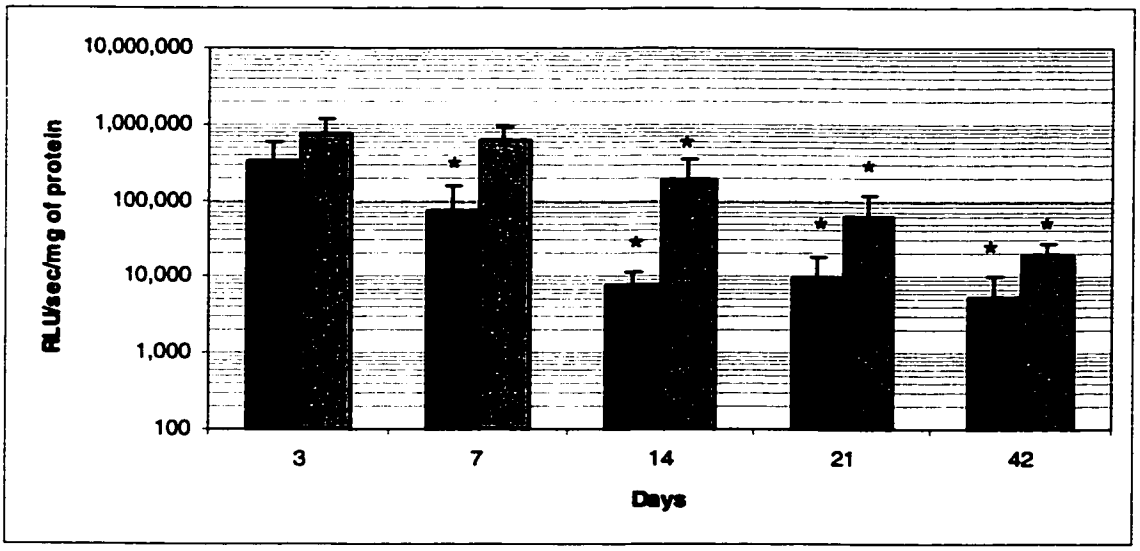
Myocyte destruction was not solely due to MHC I restricted perforin mediated lysis.

Pfn K/O (H-2^b) mice, as the name suggests, lack the gene for perforin and therefore are deficient in their ability to induce perforin-mediated lysis by antigen-specific CD8+ T-cells. When 50μg of the HBs/luciferase colinear-expression vector was delivered to the TA muscles of these animals, a decrease to about 2% (relative to the 3-day level) of luciferase expression was observed by 14 days (Fig 7).

To eliminate CD8+ T cells completely from the immune equation, 50μg of the HBV S expressing co-expression vector was delivered to C1D mice (also based on H-2^b), which lack any functional β2-microglobulin resulting in the absence of functional MHC class I molecules. These animals lack mature CD8+ T cells (187), and should therefore display a severe deficiency in any CD8+ T cell mediated processes, whether mediated by perforin or not. In these animals the expression of luciferase was reduced over time (Fig 7), similar to that seen in the in the normal C57BL/6 H-2^b control mice (Fig. 6B), however the decrease

Figure 7. Myocyte destruction was immune mediated but not solely due to an MHC I restricted, perforin mediated lysis. 50μg of the HBsAg/luciferase co-linear expression vector was delivered IM to the TA muscle of mice with different immunocomprimised backgrounds: SCID mice (H-2^d), MHC class II deficient mice (C2D) (H-2^b), perforin Knockout mice (Pfn K/O) (H-2^b), and MHC class I deficient mice (C1D) (H-2^b). TA muscles (n=10) were removed at different time points post injection. The data is represented as means ± SD with *=$p<0.01$ when compared to the day 3 values.


1,000,000
100,000
10,000
1,000
100
RLU/sec/mg of protein
3
7
14
21
42
Days


10,000,000
1,000,000
100,000
10,000
1,000
100
RLU/sec/mg of protein
3
7
14
21
42
Days

occurred somewhat later and to a lesser degree than that seen in the Pfn K/O mice (Fig 7).

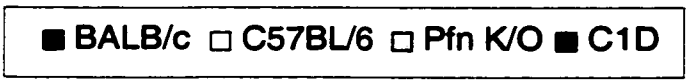
Myocyte destruction was dependent on MHC II restricted CD4+ T cell activation.

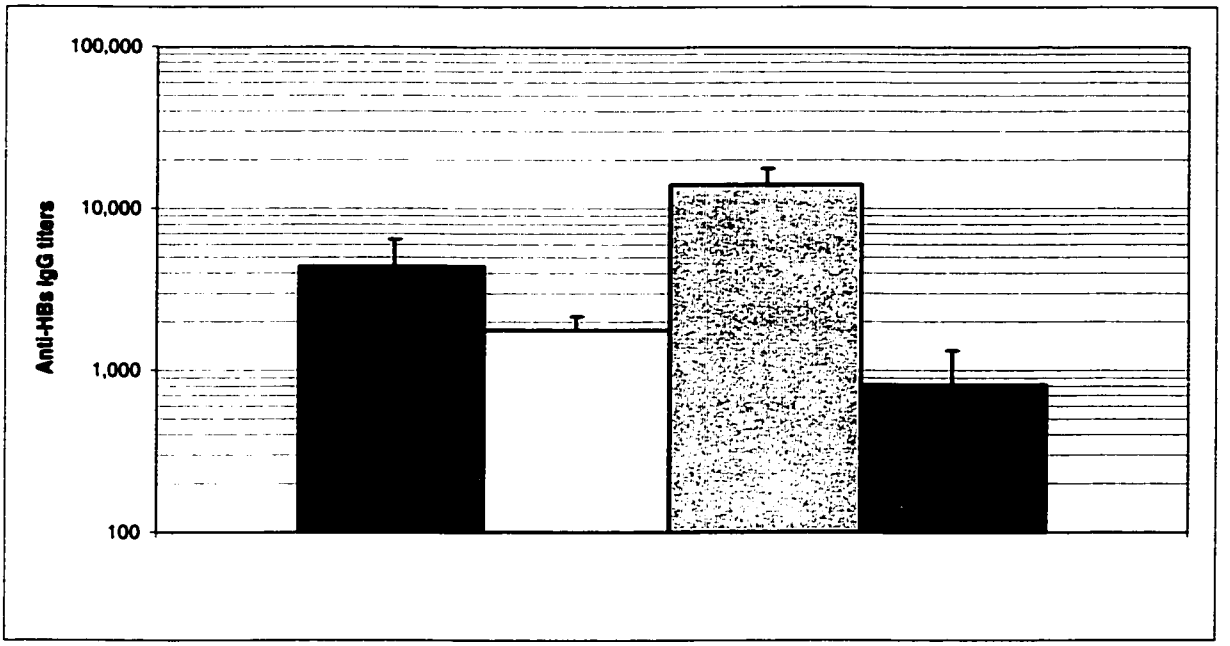
To evaluate the influence of CD4+/MHC II restriction on the destruction of muscle cells following DNA immunization, 50μg of the HBV S expressing co-expression vector was delivered to TA muscles of C2D mice (also based on H-2^b). C2D mice are knockout mice that do not express any functional MHC class II molecules and do not have any mature CD4+ T cells (188), which are required for induction of both humoral and cell-mediated adaptive immune responses. In the absence of these immunological parameters, no significant decrease in luciferase expression was observed at any time point, as had been the case in SCID mice (Fig 7).

Myocyte destruction was associated with anti-HB antibody production.

In addition to evaluating the level of luciferase expression as a marker for muscle destruction, the production of anti-HBs antibodies was also explored. In all cases where there was a significant decrease in luciferase expression (associated with the expression of the HBV S protein), there was also a detectable anti-HBs antibody response (Fig 8).

Figure 8. The destruction of myocytes was associated with anti-HBs antibody production. At 42 days post injection, plasma was collected from all groups of mice (n=5) and their titre of anti-HBs IgG evaluated by ELISA. Anti-HBs antibodies were undetectable in the BALB/c control, C57BL/6 control, SCID, and C2D mouse groups. The Data are represented as the mean±SEM.




100,000
10,000
1,000
100
Anti-HBs IgG titers

3.3. HBV chimpanzee studies.

In an effort to move from rodent models towards human use, the influence of an HBV DNA vaccine and/or the combination of CpG ODN with a currently available HBV vaccine on immune activation in chimpanzees was evaluated.

Anti-HBs antibody production generated following the different vaccination strategies in chimpanzees.

Groups of two animals received injections at 0 and 4 weeks of one of (i) Engerix B, (ii) Engerix B + CpG ODN, or (iii) an HBsAg expressing DNA vaccine. The combination of a DNA vaccine at 0 weeks and Engerix B + CpG ODN at 4 weeks was also investigated. Anti-HBs levels were evaluated from plasma on a bi-weekly basis by ELISA and expressed in mIU/mL (Table 1). The minimum anti-HBs level required for protection from natural infection is 10 mIU/mL (69-72). The CpG ODN/Engerix B vaccine combination resulted in a more effective induction of immune activation achieving protective antibody levels of 94 and 127 mIU/mL only 2 weeks following the first vaccine dose. In contrast, Engerix B alone achieved antibody levels of only 11 and 6 mIU/mL at 4 weeks following the first vaccine dose. The animals receiving two doses of the HBsAg expressing DNA vaccine did not develop any detectable anti-HBs antibodies prior to challenge. The animals that received the DNA vaccine followed by the CpG ODN/Engerix B combination did develop detectable anti-HBs antibodies prior to challenge but only following the second vaccine dose. An attempt was also made to evaluate

Table 1. Anti-HBs antibody levels, in mIU/mL, generated by different vaccination strategies. The animals received injections at 0 and 4 week with one of: Engerix B, Engerix B + CpG ODN, HBsAg expressing DNA vaccine, or the combination of the DNA vaccine at 0 weeks and Engerix B + CpG ODN at 4 weeks. Anti-HBs levels were determined from plasma collected bi-weekly using the Monolisa anti HBS 3.0 commercial ELISA kit (Sanofi Pasteur).

		Weeks				
Vaccines	Animals	0	2	4	6	8
Engerix B + CpG	5960	0	94	135	16,800	8,243
Prime and boost	1581	0	127	125	9,640	1,322
Engerix B	5855	0	0	11	4,706	809
Prime and boost	1580	0	0	6	3,712	796
DNA vaccine	1573	0	0	0	0	0
Prime and boost	1574	0	0	0	0	0
DNA vaccine prime	1578	0	0	0	569	194
Engerix B + CpG boost	1579	0	0	0	154	51
Infection control	5872	0	0	0	0	0

differential antibody isotype production in response to the different vaccination strategies in an effort to identify possible propensities for differential Th cell development. The only Ig isotype detectable in the animals that developed an anti-HBs antibody response prior to challenge was IgG1 (data not shown).

The outcome of infectious HBV challenge following different vaccination strategies in chimpanzees.

The animals were challenged IV at 8 weeks with infectious HBV and evaluated on a weekly basis for the presence of productive infection, which was indicated by the presence or absence of HBsAg and HBeAg in the plasma (Table 2). The presence of both antigens in the plasma serves as a marker of productive infection. As a positive control for infection, animal 5872 received only saline throughout the vaccination protocol and became positive for both HBsAg and HBeAg by 7 weeks post challenge. However, all animals that had developed protective level of anti-HBs antibodies were protected from challenge. Interestingly, one of the DNA vaccinated animals (No.1573) was also protected from challenge even in the absence of any detectable pre-challenge anti-HBs antibodies.

Therefore, the presence of anti-HBs antibodies in animal 1573 was evaluated post challenge in an effort to explain the existence of protection in the absence of detectable pre-challenge anti-HBs antibodies (Table 3). Animal 1573 developed protective levels of antibodies following challenge sooner than the animals 1574 and 5872, both of which developed productive infections.

Table 2. Evaluation of productive infection by HBV post challenge as indicated by the presence of circulating HBsAg/HBeAg. Animals were challenged IV at 8 weeks with $10^{3.5}$ CID_{50} of HBV strMS-2. The presence of HBsAg and HBeAg as indicators of productive HBV infection was assessed, on weekly plasma samples, by commercial ELISA (ABBOTT)

		Weeks post challenge					
Vaccines	Animals	0 - 5	6	7 - 11	12 - 14	15 - 18	19 - 24
Engerix B + CpG	5960	-/-	-/-	-/-	-/-	-/-	-/-
prime and boost	1581	-/-	-/-	-/-	-/-	-/-	-/-
Engerix B	5855	-/-	-/-	-/-	-/-	-/-	-/-
prime and boost	1580	-/-	-/-	-/-	-/-	-/-	-/-
DNA vaccine	1573	-/-	-/-	-/-	-/-	-/-	-/-
Prime and boost	1574						
DNA vaccine prime	1578	-/-	-/-	-/-	-/-	-/-	-/-
Engerix B + CpG boost	1579	-/-	-/-	-/-	-/-	-/-	-/-
Infection control	5872						
		HBsAg/HBeAg					

Table 3. Anti-HBs antibody levels, in mIU/mL, following challenge at 8 weeks. Post-challenge anti-HBs levels were assessed on bi-weekly plasma samples using the Monolisa 3.0 anti HBS commercial ELISA kit (Sanofi Pasteur).

		Weeks Post Challenge							
Vaccines	Animals	2	4	6	8	10	12	14	16
DNA vaccine	1573	0	23	77	39	24	15	17	15
Prime and boost	1574	0	0	0	0	45	0	0	0
Infection control	5872	0	0	0	0	0	74	14	0

HBsAg-specific T cell responsiveness generated following the different vaccination strategies in chimpanzees.

HBsAg-specific induction of T cell proliferation was evaluated on a bi-weekly basis by a standard *in vitro* T cell proliferation assay (Table 4). All vaccination strategies resulted in the development of HBsAg-specific T cell proliferation by either 4 or 6 weeks of the vaccination schedule. Of note was the detection of HBsAg-specific T cell proliferation in animals 1573 and 1574, both of which received only the DNA vaccine and did not develop any detectable pre-challenge anti-HBs antibodies.

Parallel to the evaluation of HBsAg-specific T cell proliferation was the assessment of HBsAg-specific cytokine production. Using a standard *in vitro* T cell proliferation assay, culture supernatants were collected following different periods of stimulation (6, 24, 48, 72hrs) with HBsAg and assessed for the presence of TNFα, IL-4, IL-5, and IFNγ by commercial ELISA. Selecting 2 weeks post the second vaccination as the best opportunity to evaluate antigen-specific T cell reactivation *in vitro*, both TNFα and IFNγ were detectable across all vaccine strategies (Table 5). However, closer examination revealed that, in some cases, animals that received some form of DNA (either DNA vaccine or CpG ODN) displayed greater and sustained increases in the production of these two cytokines when re-stimulated with varying concentrations of HBsAg. In contrast, neither IL-4 nor IL-5 was detectable following any of the vaccination strategies (data not shown).

Table 4. HBsAg-specific T cell proliferation generated by different vaccination strategies. PBMCs were isolated bi-weekly and stimulated for 6 days with HBsAg (5, 2.5, and 1.25 μg/mL) or PHA (10 μg/mL), then pulsed with ^{3}H-thymidine at 1 μCi/well for 18 hours. T-cell proliferation was expressed as a stimulation index. A sample was considered positive when the stimulation index of the three HBsAg concentrations tested was greater than 4.

Weeks of study	Animal 5960 CpG + Engerix B				Animal 1581 CpG + Engerix B			
	HBsAg Protein (ug/mL)			PHA (ug/mL)	HBsAg Protein (ug/mL)			PHA (ug/mL)
	1.25	2.5	5	10	1.25	2.5	5	10
0	2.1	1.7	0.8	11.4	3.6	2.8	2.6	67.9
2	1.8	2.1	1.4	4.9	5.8	2.5	2.9	16.2
4	0.6	2	2.1	6.5	3.6	5.1	3.5	7.2
6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
8	2.2	1.8	1.7	17.3	2.7	3.6	2.4	59.8

Weeks of study	Animal 5855 Engerix B				Animal 1580 Engerix B			
	HBsAg Protein (ug/mL)			PHA (ug/mL)	HBsAg Protein (ug/mL)			PHA (ug/mL)
	1.25	2.5	5	10	1.25	2.5	5	10
0	1.2	1	0.9	8.3	2.4	1.8	0.7	24.5
2	2.3	1.7	1.7	2.5	2.8	2	2.3	5.3
4	3.8	4.4	5.3	27	[illegible]	[illegible]	[illegible]	[illegible]
6	[illegible]	[illegible]	[illegible]	[illegible]	2.4	3	1.8	3.9
8	[illegible]	[illegible]	[illegible]	[illegible]	2.1	1.7	3.5	17.7

Weeks of study	Animal 1573 DNA vaccine				Animal 1574 DNA vaccine			
	HBsAg Protein (ug/mL)			PHA (ug/mL)	HBsAg Protein (ug/mL)			PHA (ug/mL)
	1.25	2.5	5	10	1.25	2.5	5	10
0	0.9	1.6	0.5	10.2	0.7	0.4	0.7	3.2
2	2.9	2	3	4.7	1.2	1	0.9	1
4	4	3.1	3.4	9.1	[illegible]	[illegible]	[illegible]	[illegible]
6	[illegible]	[illegible]	[illegible]	[illegible]	1.3	1.2	0.9	4.1
8	[illegible]	[illegible]	[illegible]	[illegible]	1.3	1	0.9	3.2

Weeks of study	Animal 1578 DNA prime/CpG + Engerix B boost				Animal 1579 DNA prime/CpG + Engerix B boost			
	HBsAg Protein (ug/mL)			PHA (ug/mL)	HBsAg Protein (ug/mL)			PHA (ug/mL)
	1.25	2.5	5	10	1.25	2.5	5	10
0	0.9	1.1	0.5	15.1	3.5	1.3	2.4	39.7
2	1.6	1.4	1.3	4.1	1.5	1.7	1.4	1.6
4	1.3	1.5	1	11.8	1.1	0.6	1.9	3.4
6	2.3	1.2	2.2	9.5	[illegible]	[illegible]	[illegible]	[illegible]
8	0.9	0.7	1.1	20.8	2.3	3.1	3.7	14.7

Weeks of study	Animal 5872 Infection control			
	HBsAg Protein (ug/mL)			PHA (ug/mL)
	1.25	2.5	5	10
0	0.9	1	0.8	4.8
2	0.9	0.9	0.7	0.9
4	0.9	1.5	1.1	2.6
6	1.7	1.5	2	2.3
8	1.5	2.1	2.6	3

Table 5. HBsAg-specific induction of TNFα and IFNγ (pg/mL) production at 2 weeks post boost. PBMCs were stimulated with HBsAg(1.25, 2.5, 5 μg/mL) or PHA (10 μg/mL). Supernatants were collected and assessed for TNFα (A) at 6hrs and IFNγ (B) at 48hrs-post stimulation by OPTEIA™ human cytokine ELISA sets (Pharmingen).

A

			HBsAg			
Vaccines	Animals	Media	5μg/mL	2.5μg/mL	1.25μg/mL	PHA
Engerix B + CpG	5960	696	>2000	1,884	1,785	1,889
Prime and boost	1581	97	757	802	721	821
Engerix B	5855	263	850	820	644	845
Prime and boost	1580	432	990	1,033	1,194	1,591
DNA vaccine	1573	245	1,414	1,206	1,449	1,727
Prime and boost	1574	732	1,666	1,648	1,271	>2000
DNA vaccine prime	1578	607	963	967	1,103	1,721
Engerix B + CpG boost	1579	165	832	708	830	1,220
Infection control	5872	1,177	>2000	1,900	1,633	>2000

B

			HBsAg			
Vaccines	Animals	Media	5μg/mL	2.5μg/mL	1.25μg/mL	PHA
Engerix B + CpG	5960	34	1,147	1,084	816	>1200
Prime and boost	1581	<10	497	463	115	>1200
Engerix B	5855	<10	267	262	278	>1200
Prime and boost	1580	<10	1,205	294	585	>1200
DNA vaccine	1573	<10	2,313	1,940	1,590	>1200
Prime and boost	1574	<10	207	154	80	>1200
DNA vaccine prime	1578	<10	837	425	160	>1200
Engerix B + CpG boost	1579	<10	182	125	98	>1200
Infection control	5872	78	646	499	302	>1200

HBsAg-specific CTL activity generated following the different vaccination strategies in chimpanzees.

In an effort to further evaluate the potential for the development of CMI, the animals were assessed for the existence of HBsAg-specific CTL activity, following the different vaccine strategies using an *in vitro* antigen-specific re-stimulation protocol and subsequent chromium release assay. Initially a vaccinia virus based system of re-stimulation and target generation failed to detect any HBsAg-specific CTL activity prior or subsequent to challenge (Table 6). However, due to concerns that high background lysis associated with the vaccinia virus based system may mask any detectable HBsAg-specific CTL activity, an alternate HBsAg peptide based system was employed. In contrast to the vaccinia virus based assay, the peptide-based assay involved the simple addition of overlapping peptides, generated from the HBV S protein, to cultures of target cells. Using this system, HBsAg-specific CTL activity was re-evaluated at 2 weeks post second immunization on cryogenically preserved cells (Table 7). This time point was chosen as the most likely time point for the detection of HBsAg-specific CTL activity following vaccination. Of the eight animals that were vaccinated, only 6 were re-evaluated for HBsAg-specific CTL activity due to a limited supply of cells for the remaining two. Of the six animals re-evaluated, animal 1581 and 1579, both of which received some form of DNA, displayed clear HBsAg-specific CTL activity. Interestingly, the specificity of the CTL activity differed between the two animals for a given pool of peptides generated from the HBV S protein. Of note was the lack of CTL activity associated with animal 1573 which failed to develop any detectable anti-HBs antibodies but displayed a

Table 6. Representative HBsAg-specific CTL activity at 2 weeks post boost using a vaccinia virus based stimulation and target system. PBMCs were isolated co-cultured with HBV S-expressing autologous EBV transformed B cells for 7 to 8 days and fed at 3-day intervals with fresh media supplemented with IL-2. On day 7 or 8, cytolytic activity was determined by standard 4-hour ^{51}Cr release assay using HBV S-expressing autologous EBV transformed B cells as targets. A positive CTL was defined as specific lysis of 10 or greater.

	Animal 5960 Engerix B + CpG			Animal 1581 Engerix B + CpG		
E:T ratio	VV-S	VV-WT	Specific	VV-S	VV-WT	Specific
6.25:1	15	10	5	22	21	1
25:1	24	21	3	43	44	0
50:1	26	27	0	51	48	3
100:1	29	33	0	56	56	0
	% Lysis					

	Animal 5855 Engerix B			Animal 1580 Engerix B		
E:T ratio	VV-S	VV-WT	Specific	VV-S	VV-WT	Specific
6.25:1	12	7	5	17	12	5
25:1	22	18	4	35	36	0
50:1	26	23	3	48	42	6
100:1	33	31	2	52	51	0
	% Lysis					

	Animal 1573 DNA vaccine			Animal 1574 DNA vaccine		
E:T ratio	VV-S	VV-WT	Specific	VV-S	VV-WT	Specific
6.25:1	19	19	0	12	7	5
25:1	35	38	0	26	21	6
50:1	46	45	1	37	26	11
100:1	55	52	2	46	41	5
	% Lysis					

	Animal 1578 DNA/Engerix B + CpG			Animal 1579 DNA/Engerix B + CpG		
E:T ratio	VV-S	VV-WT	Specific	VV-S	VV-WT	Specific
6.25:1	12	7	5	25	22	3
25:1	22	18	4	45	45	0
50:1	32	26	5	54	58	0
100:1	35	33	3	67	68	0
	% Lysis					

Table 7. HBsAg-specific CTL activity at 2 weeks post boost using an HBV S peptide based system. Altering the CTL assay to incorporate a peptide based system of stimulation, cryogenically stored PBMCs were rapidly thawed, and cultured with pooled synthetic HBV S peptides for 14 days. Cultures were fed at 3-day intervals with fresh media supplemented with IL-2, and on day 7 the cultures were restimulated with the synthetic peptides. On day 14 the cytolytic activity was determined with a standard 4-hour ^{51}Cr release assay using autologous EBV transformed B cells, pulsed with synthetic HBV S peptides, as targets. A positive CTL is defined by a specific lysis of 10 or greater. The absence of animals 5855 and 1574 reflects a limitation in the cell number available for retest.

	Animal 5960 Engerix B + CpG		Animal 1581 Engerix B + CpG	
E:T ratio	Pool B	Pool C	Pool B	Pool C
6.25:1	1	2	0	3
12.5:1	3	2	0	8
25:1	1	3	0	11
50:1	2	3	1	19
	% specific Lysis			

	Animal 1580 Engerix B	
E:T ratio	Pool B	Pool C
6.25:1	0	1
12.5:1	0	1
25:1	1	2
50:1	2	1
	% specific Lysis	

	Animal 1573 DNA Vaccine	
E:T ratio	Pool B	Pool C
6.25:1	2	2
12.5:1	10	0
25:1	2	2
50:1	2	4
	% specific Lysis	

	Animal 1578 DNA prime/Engerix B + CpG boost		Animal 1579 DNA prime/Engerix B + CpG boost	
E:T ratio	Pool B	Pool C	Pool B	Pool C
6.25:1	0	3	3	1
12.5:1	2	0	6	2
25:1	2	1	12	1
50:1	1	4	26	0
	% specific Lysis			

strong HBsAg-specific T cell proliferative response and cytokine production and was protected from challenge.

4. Discussion

4.1. The need for an effective HBV therapeutic vaccine

Effective control of HBV infection has been linked to the development of a strong antigenically diverse immune response characterized by good CMI (42). In the absence of this response, resolution does not occur and a persistent viral infection ensues. The consequence of persistent infection is the eventual development of chronic hepatitis, which leads to the development of secondary liver sequelae such as liver cirrhosis and HCC (41). Currently, it is estimated that more than 350 million people worldwide are persistently infected with HBV (41). This represents large populations of persistently infected individuals and constitutes enormous reservoirs of infectious virus. The development of secondary sequelae in these populations, associated with chronic liver disease, places a heavy burden on health care systems throughout the world.

Prophylactic vaccines against HBV do exist but are only recently being widely used in highly endemic countries (14). These vaccines overall are extremely effective for prophylaxis, however they do fail to protect 5-15% of healthy vaccine recipients (69-72), but more relevant to the topic of this thesis, they have demonstrated extremely limited potential for therapeutic use (189). The draw-back of alum-adjuvanted protein vaccines, in particular for therapeutic applications, is that they fail to effectively induce a CMI response (131, 161), and it is clear that an HBV-specific CMI response is what is lacking in chronically infected individuals (46).

This body of work attempted to evaluate the potential of using novel approaches to immunize against HBV that might prove to be better for prophylactic vaccination, but more importantly may be effective as therapeutic regimes for chronic HBV infection. Specifically, two types of novel HBV vaccines that induce Th1-type immunity were evaluated, (i) recombinant HBsAg with CpG ODN as an adjuvant and (ii) HBsAg-expressing DNA vaccines. The quality of the immune responses that are induced by these vaccine strategies, including the development of CMI, supports their potential to overcome hypo or non-responsiveness to HBsAg in healthy vaccine recipients and to various HBV proteins in chronically infected patients.

4.2. Influence of CpG ODN on immune activation in HBsAg-transgenic mice.

Initial experiments were performed in an HBsAg transgenic mouse model in an effort to extend previous observations made with the DNA vaccine to CpG ODN. These experiments revealed that, like the DNA vaccine, immunization of the HBsAg Tg mice with CpG ODN and recombinant S2.S was capable of mediating the non-cytopathic control of HBsAg transgene expression. This was visualized by a loss of circulating HBsAg, the generation of anti-HBs antibodies, and a reduction of transgene mRNA content (190). Pathology has been previously shown to be absent during this process (172). The control of transgene expression was not observed when animals received recombinant S2.S in combination with the non-CpG ODN (190).

Of interest was the observed difference in the kinetics of transgene regulation in response to a single administration of the DNA vaccine versus three monthly administrations of the S2.S + CpG ODN combination. In an effort to understand this observed difference, innate and HBsAg-specific immune responses were evaluated in the transgenic as well as congenic normal control C57BL/6 mice.

***Influence of CpG ODN on innate immune activation of C57BL/6 splenocytes* in vitro.**

When spleen cells were taken from naïve C57BL/6 mice and stimulated *in vitro*, CpG ODN was superior to media, control non-CpG ODN or *E. coli* DNA at inducing splenocyte proliferation. The *E. coli* DNA was to serve as a positive control for CpG DNA mediated stimulation, but failed to give a stimulation index greater than media alone (87). The absence of measurable stimulation in association with *E. coli* DNA may have been related to its phosphodiester backbone. Native DNA backbone is much more sensitive to nucleases than the synthetic phosphorothioate backbone used in the synthesis of the ODNs. Therefore, degradation of the *E. coli* DNA, in culture, may have led to a pool of available nucleotides that competed with the radiolabeled thymidine used as the indicator of cellular proliferation. However, visual observation of the cultures did not reveal a detectable outgrowth of cells, as was the case with the CpG ODN. This suggests that, even in light of the possible limitations of the thymidine incorporation assay with respect to the *E.coli* DNA, the CpG ODN was superior to *E.coli* DNA with respect to the induction of cellular proliferation. This is not unexpected given the high concentration of CpG motifs present in the CpG ODN

relative to native *E. coli* DNA in combination with the nuclease resistant backbone.

CpG ODN was also superior to the non-CpG ODN at inducing the production of TNFα, IL-12, IL-6, and IFNγ. Interestingly, despite an apparent lack of proliferation, *E.coli* DNA was as effective as the CpG ODN at inducing the production of IL-12 and IFNγ, though less effective at stimulating TNFα, and IL-6 production. These observations support the hypothesis that CpG motifs, either contained in CpG ODN or *E. coli* DNA can mediate a non-antigen-specific stimulation of C57BL/6 splenocytes.

Although the observed ability of CpG DNA to stimulate murine splenocytes is consistent with the literature (87), the absence of proliferation and IL-6 production noted with *E.coli* DNA may be associated with the propensity of C57BL/6 mice towards a Th1 bias. This may influence their ability to produce IL-6 and in turn influence splenocyte proliferation, since the majority of cells that actually proliferate following *in vitro* stimulation of mouse splenocytes with CpG ODN are B cells (85). Therefore it is possible that, unlike the CpG ODN, the *E. coli* DNA was simply less efficient at overcoming this Th1 influence for reasons likely related to differences in molecular characteristics between *E. coli* DNA and CpG ODN.

Influence of CpG ODN on the generation of an HBsAg-specific humoral immune response in C57BL/6 mice.

To evaluate the influence of CpG ODN on antigen-specific immune responses in the C57BL/6 mice, CpG ODN or non-CpG ODN was combined with recombinant S2.S and delivered in three monthly immunizations. For the purpose of comparison to the observations made in the transgenic animals, a third group of C57BL/6 mice received a single immunization with an S2.S encoding DNA plasmid. When evaluating the HBsAg-specific humoral immune response associated with each vaccination strategy, it was observed that animals immunized with the combination of CpG ODN and S2.S developed significantly higher total IgG antibody titers than did animals immunized with S2.S + non-CpG ODN or the DNA vaccine. Interestingly, the level of total IgG antibody titers generated in the animals that received the non-CpG ODN with S2.S was not significantly higher than the levels from the animals that received the DNA vaccine. The Th bias could not be assessed by comparison of antigen-specific antibody isotype levels because of the lack of the IgG2a gene in the C57BL/6 mouse (183-185). Although differences in total IgG levels between the different vaccine strategies was not unexpected, considering the animals that received the S2.S + ODN combinations were immunized three times versus the single immunization with the DNA vaccine, these data do allow for some interesting speculation. The combination of S2.S and non-CpG ODN demonstrated the ability to generate relatively good antibody production, when you consider that antigen alone generally fails to generate titers greater then 100 (190), suggesting the possible presence of adjuvant properties other than CpG motifs. In fact this

has been documented in the literature, and attributed largely to the relatively weak adjuvant properties of the phosphorothioate backbone, as well as other immunostimulatory motifs such as poly-G's (191). However, the ability to induce HBsAg-specific antibodies was not alone able to control transgene expression. Although all the vaccine strategies were capable of inducing antibody production in the C57BL/6 mice, only the DNA vaccine and the S2.S + CpG ODN combination were capable of efficiently breaking immune tolerance in the transgenic animals and controlling transgene expression (190).

Influence of CpG ODN on the generation of an HBsAg-specific CTL response in C57BL/6 mice.

When evaluating the HBsAg-specific CTL response associated with each vaccination strategy, it was observed that C57BL/6 mice immunized with the combination of CpG ODN and S2.S, either once or three times, did not generate any detectable HBsAg-specific CTL activity. In contrast, C57BL/6 mice that were immunized only once with the DNA vaccine generated a clear HBsAg-specific CTL response. This differential induction of CTL has been observed in other mouse strains (unpublished observations) and suggests a possible explanation for the differential kinetics observed for the control of transgene expression induced by the different vaccine strategies in the congenic Tg mice.

Therefore the data presented herein suggests that DNA vaccination was more efficient at inducing the appropriate CMI response required for the control of transgene expression. Therefore, the differential kinetics observed during the

regulation of transgene expression may have been associated with a difference in the strength of the CMI response induced by the different vaccine strategies.

Influence of CpG ODN on the generation of HBsAg-specific IFNγ secreting T cells in C57BL/6 mice.

Given the importance of IFNγ production in the development of CMI (186) and in the non-cytopathic control of HBV gene expression (56, 58, 173), the *in vitro* HBsAg-specific IFNγ production was evaluated in association with each vaccine strategy. Splenocytes from immunized C57BIL/6 mice were re-stimulated *in vitro* with HBsAg. Significantly higher levels of IFNγ were produced by splenocytes taken from C57BL/6 mice that received a single dose of DNA vaccine as compared to C57BL/6 mice that received a single doses of the S2.S + CpG ODN combination.

The differential induction of HBsAg-specific INFγ secreting cells was also observed by ELISPOT assay. Splenocytes collected from C57BL/6 animals that had been vaccinated either twice at monthly intervals with the HBsAg + CpG ODN combination or once with the DNA vaccine, were evaluated for the presence of HBsAg specific IFNγ secreting T cells by ELISPOT analysis (190). Similar to the responses evaluated using culture supernatants, these results underline the enhanced ability of the DNA vaccine at generating HBsAg-specific IFNγ secreting T cells (190).

Subsequently, splenocytes from the immunized C57BL/6 mice were enriched for CD8+ T cells via negative selection then adoptively transferred into the Tg

animals (190). The CD8+ T cell enriched splenocytes, taken from animals immunized twice with the S2.S + CpG ODN combination, were not as effective at eliminating circulating HBsAg or suppressing transgene mRNA expression, as those taken from animals that were immunized once with the DNA vaccine (190). This therefore further supports the differential capacity of each vaccine strategy to induce an effective HBsAg-specific CD8+ T cell response.

Collectively these observations suggest that the increased efficiency at which the DNA vaccine is capable of inducing an HBsAg-specific CMI response accounts for the differential regulation of transgene expression observed with the two vaccine strategies.

Summary

This study has demonstrated that, like the DNA vaccine, the combination of S2.S + CpG ODN was capable of breaking immune tolerance and negatively regulating transgene expression in an HBsAg transgenic mouse model, highlighting its potential as a therapy for chronic hepatitis B. The study also revealed that the Th1 quality and magnitude of the immune response was the crucial variable for control of transgene expression in this HBsAg transgenic mouse model, since the non-CpG ODN failed to break tolerance. Similar observations have been reported in other transgenic mouse models and following natural infections in humans (45, 46, 58). Therefore, this study highlights a fundamental difference between these two vaccine strategies, the

capability of generating immune responses, in mice, of similar quality but of different magnitudes, possibly related to different mechanisms of action.

4.3. Relation between immune activation and the longevity of antigen expression following DNA vaccination.

To further explore the superiority of the DNA vaccine over the protein + CpG ODN based vaccine at inducing antigen-specific CMI response, the relationship between immune activation and the longevity of antigen expression following DNA immunization was evaluated.

The loss of antigen expressing myocytes following DNA vaccine is immune mediated

Our lab had previously observed that the loss of luciferase expression was associated with an antigen-specific immune-mediated destruction of transfected myocytes (167), and this was confirmed in the present study, except using a colinear expression vector rather than two different vectors to express the antigen and luciferase. In contrast to the non-HBV S expressing control vector, following delivery of the HBV S/luciferase co-linear expression vector to immune competent BALB/c and C57BL/6 mice, there was a significant decrease in luciferase expression over time, as previously observed (167). Furthermore, when the HBV S/luciferase co-linear expression vector was delivered to SCID mice, no significant decrease in luciferase expression was observed over time. The significant decrease in luciferase expression at 42 days associated with the control vector was likely due to the induction of weak, slowly developing, luciferase-specific immune responses. Although this was not observed in the

previous study (167), a luciferase-specific immune response has been reported by others (192). In addition, the observation that luciferase expression is stably maintained in the SCID mice throughout the study period supports this hypothesis.

The faster kinetics and greater magnitude of muscle destruction observed in BALB/c as compared to the C57BL/6 mice, is likely related to differences in H2-restricted recognition of T cell epitopes on the HBsAg and luciferase antigen (193).

Influence of perforin independent immune responses on the longevity of antigen expression following DNA vaccination.

Since a DNA vaccine works by virtue of intracellular synthesis of antigenic proteins that are expressed in the context of MHC class I, the role of CD8+ T cell/MHC I restricted and perforin-mediated destruction of myocytes was assessed. When the HBV S/luciferase co-expression vector was delivered to Pfn K/O mice ($H\text{-}2^b$), which do not express functional perforin, a decrease in luciferase expression was observed, and surprisingly this occurred sooner and was stronger than that seen in the MHC matched parental C57BL/6 strain. It has been traditionally accepted that CD8+ T cells mediate cellular destruction via a perforin- and granzyme-dependent induction of apoptosis (194). However, perforin independent mechanisms of cytotoxicity through CD95/CD95L have recently been described (194). In addition, indirect cytokine mediated effects may play a role in this system through the inhibition of viral replication and the induction of apoptosis (194). Recently it has been suggested that perforin may

be more than just an effector molecule (195). It may play a regulatory role in the generation of certain immune effector functions, such as antibody production (195). This could explain the increase in kinetics and magnitude of myocyte destruction as compared to the parent strain.

Regardless, these data suggest that destruction of antigen-expressing myocytes following DNA vaccination occur, at least in part, by perforin-independent mechanisms.

Influence of CD8+ T cell independent immune responses on the longevity of antigen expression following DNA vaccination.

The role of CD8+ T cell-independent mechanisms in the immune mediated destruction of myocytes was evaluated in C1D mice that lack functional MHC I molecules and ultimately mature CD8+ T cells. A significant decrease in luciferase expression was also observed in these mice. The kinetics of this response was similar to that displayed by the MHC matched parental C57BL/6 strain but the magnitude was less. Myocyte destruction, in this environment, may be due to antibody dependent mechanisms of cellular lysis. There was a strong correlation between the development of an anti-HBs antibody response and myocyte destruction. HBsAg is expressed as a transmembrane protein (196-198), which makes it accessible to circulating antibodies. Therefore two well-documented mechanisms of antibody-dependent cellular cytotoxicity (ADCC) could play an important role in HBsAg restricted cellular lysis, namely: (i) complement mediated ADCC (199) and (ii) Fc receptor-mediated ADCC (200).

Therefore, a CD8+ T cell/MHC I-restricted mechanism of cellular cytotoxicity cannot solely explain antigen-specific immune-mediated destruction of myocytes.

Influence of CD4+ T cell/MHC II restriction on the longevity of antigen expression following DNA vaccination.

The contribution of CD4+ T cell/MHC II restriction on myocyte destruction was assessed in C2D mice that lack functional MHC II molecules and mature CD4+ T cells. The immune-mediated destruction of luciferase and HBsAg-expressing myocytes was completely abrogated in the C2D mice, as was observed in the SCID mice, underlining the crucial role of CD4+ T cells and/or MHC II-restricted professional antigen presentation in the development of a DNA vaccine-induced immune response. In the absence of MHC II and ultimately CD4+ T cells, the induction phase of either humoral or cellular immune activation does not occur (201, 202).

Potential of antigen expressing APCs as targets for immune mediated destruction.

Although bone marrow-derived APCs are absolutely crucial for function of DNA vaccines, controversy remains as to the role, and possibly the necessity of direct transfection by plasmid DNA. There is strong evidence in the literature to support the theory that professional APCs present exogenous antigens in the context of MHC class I (203-205). Thus eliminating the need for direct transfection, as demonstrated by Ulmer et al.(159, 160). However, many groups have reported that, following DNA vaccination, plasmid DNA travels to the draining lymph nodes. This suggests that plasmid DNA is either carried by

directly transfected APC from the site of injection, or by lymphatics which can then lead to APC transfection in the nodes (206, 207). Therefore, following DNA vaccination there could exist two populations of APC that may be involved in the activation of the immune system. The first would be the APC that are directly transfected with the DNA plasmid and which produce soluble antigen and present antigen in the context of MHC I and II. The second population would consist of APC that are not directly transfected yet are capable of antigen uptake either directly from the immediate environment or indirectly via Fc receptor-mediated uptake of antigen-antibody complexes, and in turn presenting it in the context of MHC I and II (203, 208).

Regardless of the scenario, both populations would constitute potential targets for MHC I restricted/CD8+ T cell-mediated lysis (209, 210). In addition, the present results also suggest that the directly transfected APC population may be susceptible to additional antibody-mediated mechanisms of cellular lysis based on the intracellular production and surface membrane expression of antigen.

Influence of plasmid DNA on the CD4+ T cell independent maturation of APCs.

Since the direct transfection of APC is likely to occur following DNA vaccination, another important observation can be made. The development of antigen-specific CTL can occur in the absence of CD4+ T cell help via a mechanism involving the activation of APC by CD4+ independent factors such as viral infection (201, 202). One factor that influences the efficacy of DNA vaccination is the content of CpG motifs in the DNA plasmid backbone (211). When

administered in the form of ODNs, with antigen, CpG motifs have been shown to activate APC directly (211) to mediate a CD4+ T cell independent induction of CTL activity (104). However, in the present study, a CD4+ independent mechanism of myocyte destruction was not observed in the C2D MHC II knockout mice. Therefore, assuming that direct transfection of APC occurred, this was unable to overcome the requirement for MHC II restricted CD4+ T cell-mediated activation of APC for the induction of CTL, as supported by the literature (158). Although CpG ODN has been demonstrated to influence the efficacy of DNA vaccination, the DNA vaccine used in this study was not optimized for CpG content, and may contain motifs that have been identified to have a negative impact on the activity of stimulatory CpG motifs (171).

Summary

This study confirms that antigen-expression from DNA vaccines is relatively short-lived, and is capable of stimulating many different immune mechanisms all of which could have a role in the destruction of transfected cells. This study also suggests that unlike CpG ODNs, which are capable of inducing CD4+ T cell independent APC maturation and CTL generation, the DNA vaccine in this study relied on CD4+ T cell help in generating CMI responses. The amplification of these responses likely results from the antigen-specific clonal expansion that occurs during the recognition and destruction of antigen expressing cells transfected following IM injection.

4.4. Influence of different CpG DNA vaccine strategies on immune activation in chimpanzees.

In an effort to move towards the evaluation of different CpG DNA vaccine strategies for the prophylactic or therapeutic treatment of HBV infections in humans, the influence of these strategies on immune activation in chimpanzees was evaluated.

Identification of primate-specific CpG motifs.

Since the immunostimulatory properties of CpG DNA have demonstrated some level of species-specificity with respect to different CpG ODNs (Krieg, unpublished observations), it was crucial to these studies that motifs be identified that were immunostimulatory to primates. Employing a series of *in vitro* based assays a panel of different CpG-containing phosphorothioate ODNs was evaluated for their ability to stimulate the activation of human NK and B cells (212). A subgroup of promising ODNs was then re-evaluated for their ability to induce the proliferation of PBMC preparations from humans, chimpanzees, and Rhesus Macaques monkeys. The data revealed that ODN 2006, a 24-mer with three CpG motifs, gave the best proliferative response for each test group (212). Therefore, ODN 2006 was chosen for use in these studies to evaluate the influence of different DNA based vaccine strategies on immune activation in chimpanzees.

Optimization of DNA vaccines for CpG content in an effort to overcome limitations observed in humans and non-human primates.

In contrast to rodent systems and veterinary applications (213), there have been relatively few DNA vaccines studies in humans and non-human primates, and these studies have yielded much less promising results (145, 148, 150-152, 214-216). The reasons for these difficulties are likely related to optimization of vaccines and delivery systems for different species (145, 217-219). Despite these hurdles, there have been some reports in which DNA vaccines have induced protective immunity in non-human primates (182, 220-224) or showed promise as a successful therapeutic agent for chronic HBV infection (225)

Since it has been demonstrated that the CpG content in a DNA vaccine can influence the outcome of immune activation (226, 227), the DNA vaccine in this study was optimized for CpG content using the primate-specific ODN 2006. The goal was to evaluate the potential of CpG DNA to overcome some of the limitations of the DNA vaccine in primate systems. The dose of the DNA vaccine in this study was chosen based on previous experience with DNA vaccines in chimpanzees (182).

Influence of different immunization strategies on the induction of an HBsAg-specific humoral response in chimpanzees.

When the vaccines were delivered by IM injection, it was observed that the addition of the ODN 2006, to the currently available HBV vaccine Engerix B, greatly improved the efficacy of this vaccine. Engerix B alone did not induce a strong anti-HBsAg humoral response until after the second vaccine dose. In

contrast, Engerix B + CpG ODN was capable of inducing the generation of a log fold greater than protective levels of anti-HBs antibodies as early as two weeks following the first vaccine dose.

The primary role of an adjuvant is the reduction in the threshold of immune activation, and these observations directly support, in chimpanzees, previous observations in mice that CpG ODN is a potent adjuvant when used in combination with alum (131). As previously mentioned, CpG ODNs reduce the threshold of immune activation through their influence on the maturation of B cells and APCs (85, 228).

Such was not the case with the DNA vaccine, which did not generate any detectable anti-HBs antibodies during the vaccination schedule and prior to challenge. When the DNA vaccine was combined with the Engerix B + CpG ODN combination in a prime/boost strategy, anti-HBs antibodies only became detectable following the second vaccine dose that consisted of Engerix B + CpG ODN. Furthermore, the level of antibodies detected was not indicative of a prior-priming event by the DNA vaccine. Collectively, these data suggest that the optimization of the DNA vaccine for CpG content was not sufficient to overcome the limitations of DNA vaccine efficacy in the chimpanzees.

Outcome of infectious HBV challenge following different vaccine strategies in chimpanzees

The chimpanzees were challenged with infectious HBV 8 weeks post-prime, and as would be expected, all animals that had generated protective levels of anti-

HBs antibodies (≥10 mIU/mL) were protected. Of particular interest, however, was the absence of a detectable productive infection in animal 1573 that had received the DNA vaccine only. This was intriguing for two reasons. The first and most obvious reason is that the animal escaped infection without having developed the minimum level of anti-HBs required for protection. One possible explanation for this may be the failure of the viral challenge to establish infection, however, this does not seem likely considering the dose of $10^{3.5}$ CID_{50} of the infectious virus and the fact that two other animals challenged at the same time with the same inoculum did develop productive infections. The more likely explanation for the observed results is the development of a sufficiently primed immune system that was capable of responding to challenge prior to the detection of a productive infection. This leads to the second reason why the data are intriguing, namely that the immune system was sufficiently primed for protection using only the DNA vaccine. This observation represents one of only a few documented instances in which a DNA vaccine has yielded complete protection following an infectious challenge in a non-human primate (220-222).

Following challenge the three animals that did not develop protective anti-HBs levels prior to challenge were evaluated for the development of anti-HBs antibodies. Animal 1573, which had received the DNA vaccine and was protected from infection in the absence of detectable anti-HBs antibodies, developed protective levels of anti-HBs antibodies much sooner than the two animals that succumbed to productive infection, supporting a role for pre-existing immunity in the protection of animal 1573 from challenge.

Influence of different immunization strategies on the induction of HBsAg-specific T cell responses in chimpanzees.

Antigen-specific immune activation, potentially induced by the different vaccine strategies, was also evaluated on the basis of antigen-specific T cell proliferation. These data demonstrated that antigen-specific T cell proliferation was detectable in at least one animal from each vaccination strategy, supporting the potential of each vaccination strategy for the induction HBsAg-specific immunity. Of particular interest was the strong T cell proliferation observed for animal 1573, which further confirms that the DNA vaccine did induce efficient immune priming in this animal. The second interesting observation was the measurable antigen-specific T cell proliferative response in animal 1574 that had also been vaccinated with the only the DNA vaccine. This suggested that animal 1574 may also have been primed for an anti-HBsAg response by the DNA vaccine alone, however this animal was not protected from challenge with infectious virus. Furthermore, the post-challenge antibody development did not support the pre-existence of immunity in animal 1574, suggesting that the level of immune induction mediated by the DNA vaccine in this animal was not sufficient to establish an effective memory response that could be boosted following subsequent exposure (229, 230). Another possibility would be the induction of low dose tolerance (231) following the second DNA vaccine dose based on the presumed low level of antigen expression associated with DNA vaccines. However the ability of the animal to eventually clear infection argues against this hypothesis.

Influence of different immunization strategies on the quality of induced HBsAg-specific T cell responses in chimpanzees.

The literature suggests a clear dichotomy in mice with respect to the quality of immune responses (i.e. Th2 vs. Th1) induced by alum-adjuvanted versus CpG ODN adjuvanted and DNA vaccines (125, 217). Therefore, an attempt was made to evaluate the quality of the immune responses induced by the different vaccines strategies used in this study. One way this can be done in mice is to evaluate the profile of antigen-specific antibody isotypes. It is well documented that in mice the production of IgG2a is associated with an IFNγ dependent class switch (232). Investigators have used this observation as a surrogate marker of Th1 like immune activation when comparing different methods of immune induction (i.e. vaccines) (131). Unfortunately, such a distinction has not been observed in humans. Nevertheless, based on the striking differences documented between alum based vaccines and CpG DNA based vaccines in mice, it was viewed as an excellent opportunity to determine whether identifiable differences would extend to chimpanzees. In the absence of chimpanzee-specific reagents, human reagents were employed to detect for the presence of total IgG, as well as IgG1, IgG2, IgG3, and IgG4. From the animals that had developed anti-HBs antibodies prior to challenge, the only IgG isotype that was strongly detectable was IgG1. Although disappointing, these observations were consistent with the literature with respect to alum based HBV vaccines in humans (233, 234), moreover, these observations reaffirmed the variable association between antibody isotypes and Th bias of the immune response in higher primates and humans.

A more direct approach of assessing the quality of an immune activation with respect to Th bias is the evaluation of T cell cytokine secretion profiles. It is widely accepted that IFNγ and TNFβ production is indicative of Th1 biased immune activation whereas IL-4 and IL-5 are indicative of Th2 biased immune activation (235). More recently, TNFα has also been observed in association with Th1 type responses (236, 237). In the present study, PBMCs were cultured in the presence or absence of antigen in parallel to the T cell proliferation assays, and collected supernatants were evaluated for the presence of TNFα, IFNγ, IL-4 and IL-5. At 2 weeks post boost, which was considered the optimal time point for the evaluation of cellular responses, all vaccine strategies displayed the capability of producing both TNFα and IFNγ, but failed to produce any detectable IL-4 or IL5. Therefore, as with the IgG isotype data, the cytokine data did not give any insight into the possible dichotomy of Th biased immune activation induced by the different vaccine strategies. The lack of IL-4, and IL-5 secretion by cells from individuals receiving alum based HBV vaccines has been reported in the literature (238, 239). With respect to TNFα, there did not seem to be any pattern to the production induced by the different vaccine strategies. This may reflect an additive non-antigen-specific production of TNFα by bystander APCs present in the culture, since APCs are the predominant source of biologically active TNFα . It is well documented that APCs in culture do become activated in a contact dependent fashion (240, 241), therefore this activation may have had an effect on the state of the PBMCs in the cultures as well as the levels of TNFα that were detected.

The evaluation of IFNγ, on the other hand, did reveal some noticeable differences. The strongest and most sustained production of IFNγ across three different antigen concentrations was observed in two animals which received some form of DNA in their vaccine, either CpG ODN or DNA plasmid, both of which corresponded with a strong antigen-specific proliferative response. Therefore in the absence of any definable dichotomy with respect to Th biased immune responses, these observations demonstrate that vaccine strategies that include some form of DNA yielded higher levels of detectable IFNγ during recall responses generated *in vitro*.

Influence of different immunization strategies on the induction of HBsAg-specific CTL responses in chimpanzees.

In addition to antibody isotypes and cytokine secretion profiles, antigen-specific CTL activity is another marker for the evaluation of the quality of induced immune responses. It is clear that antigen-specific CTL activity is generated within immune responses that include a CMI/Th1 component (186), and in the case of HBV infection such a response is believed to be crucial for the efficient control of viral replication (56) (58, 173). The presence of antigen-specific CTL activity following immunization was evaluated for each vaccine strategy. Initial attempts using a recombinant vaccinia virus vector system did not yield any detectable antigen-specific CTL activity. However, the high level of non-specific background associated with this assay may have masked any potential response.

To eliminate the influence of high background, a peptide-based assay for the stimulation and expansion of CTL precursors was employed. When

cryogenically stored PBMCs were evaluated, antigen-specific CTL activity was generated in two animals that had received some form of DNA in their vaccine regime. Further, the genetic diversity of these animals was highlighted by their differential reactivity to particular pools of peptides used in the assay. Unfortunately, due to a limitation in the number of cells available, two animals could not be re-evaluated for the presence of antigen-specific CTL activity. Therefore, although it cannot be concluded from this study that the alum adjuvanted vaccine strategy failed to generate any detectable CTL activity, it was clear that the DNA based vaccine strategies were capable of generating detectable antigen-specific CTL activity.

Summary

This study supports the potential of HBsAg + CpG ODN and plasmid DNA based vaccines as potentially better alternatives to existing prophylactic regimes for HBV vaccination. In addition, the quality of the generated immune responses as assessed by IFNγ production and the generation of antigen-specific CTL response supports the potential of these vaccine strategies as a therapy for chronic HBV infection. Although it may be argued that the DNA vaccine demonstrated only marginal efficacy with respect to measurable markers of immune activation, there is no arguing the ability of the DNA vaccine to generate protective immunity in one of the two chimpanzees. In the scope of DNA vaccines, this is an important observation since it is in direct support of the basic concepts of DNA vaccines. Further, these observations suggest that the limited

success that has been achieved to date in higher primates and humans reflects the hurdles of optimization rather than a weakness in the technology.

4.5. Conclusion

HBV infection remains an important global concern. More than 2 billion people worldwide have been infected by HBV with 350 million currently having persistent or chronic infection (14). The significance of chronic infection is the increased risk of developing liver cirrhosis and HCC (15). This is an important health concern in endemic regions of the world where the majority of HBV infection is acquired neonatally and is generally associated with viral persistence. Large populations of persistently infected individuals constitute enormous reservoirs of infectious virus. Many of these individuals go on to develop and succumb to the secondary sequelae, associated with chronic liver disease, placing a heavy burden on health care systems. Although currently available prophylactic vaccines are effective in most healthy individuals, they fall short in two respects. These vaccines do not effectively immunize 5-15% of healthy individuals and they do not appear to be highly effective, if at all, for the treatment of chronic disease due in part to their inability to efficiently stimulate CMI.

This work evaluated the potential of protein + CpG ODN and DNA vaccines to be used as prophylactic or therapeutic agents for the protection against or treatment of HBV infection. The mouse studies demonstrated that protein + CpG ODN and DNA vaccines were capable of overcoming tolerance to HBV in an HBV transgenic mouse model. Although far removed from human disease, these studies give an indication of the potential of these agents in altering the non-

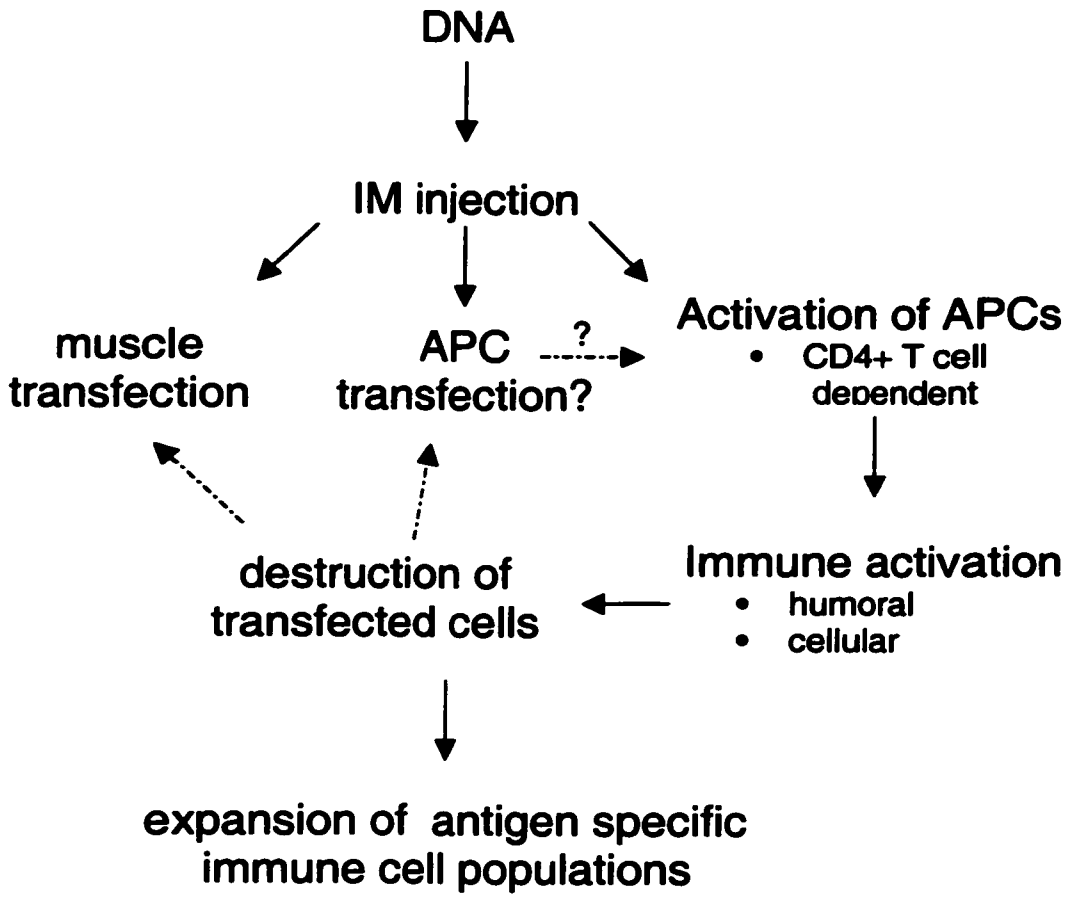
responsiveness of the immune system primarily through the induction of a strong CMI response.

The superiority of the DNA vaccine to the protein + CpG ODN vaccine in the mouse systems appears to be related to the ability of the DNA vaccine to induce a stronger CMI response. The evaluation of the relation between immune activation and antigen expression following DNA vaccination revealed that DNA transfected cells that subsequently express antigen became legitimate targets for immune mediated destruction by multiple mechanisms. This suggests that, in addition to a more efficient antigen presentation, the presence of antigen expressing cell populations may allow for the additional expansion of activated immune cell populations thereby generating a potentially larger pool of antigen-specific T cells (Fig 9A). Although protein + CpG ODN can influence the activation of CMI in part through the CD4+ T cell independent maturation of APCs (104), it does not cause as efficient an expansion of T cell populations as postulated for the DNA vaccine (Fig 9 B). This is likely due to the lower number of potential antigen expressing target cells generated following immunization with protein + CpG ODN.

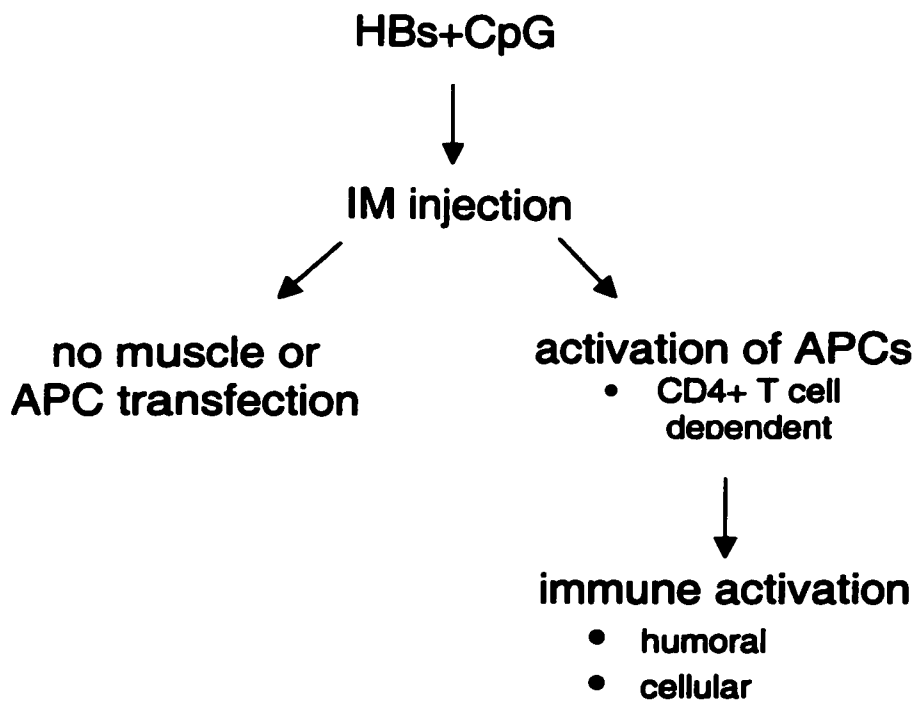
Often the prelude to human clinical trials is the demonstration of some level of efficacy in non-human primates. Since HBV's limited tropism does extend to the great apes, it makes them extremely valuable for the study of HBV vaccinology. The use of chimpanzees in this study as an HBV infection model revealed that both Engerix B (HBsAg + alum) + CpG ODN and the HBsAg-expressing DNA vaccine were capable of influencing the immune system efficiently to provide

Figure 9: Differences that may account for the differential induction of CMI by the DNA vaccine and HBsAg + CpG ODN in mice. (A) DNA vaccination leads to a population of transfected cells that express antigen, possibly on the surface, as well as in the context of MHC. These cells become legitimate targets for antigen-specific immune responses. Therefore, in addition to a more efficient antigen presentation, the destruction of antigen expressing cell populations allows for the expansion of activated T cell populations. (B) The HBsAg + CpG ODN vaccine influences the activation of CMI, in part, through the CD 4+ T cell independent maturation of APCs, which does not appear lead to an as efficient expansion of T cell populations as postulated for the DNA vaccine.

A)


DNA
IM injection
muscle transfection
APC transfection?
?
Activation of APCs
• CD4+ T cell dependent
destruction of transfected cells
Immune activation
• humoral
• cellular
expansion of antigen specific immune cell populations

B)


HBs+CpG
IM injection
no muscle or APC transfection
activation of APCs
• CD4+ T cell dependent
immune activation
• humoral
• cellular

protection from infection with HBV. In addition, evaluation of the quality of the immune responses generated by the Engerix B + CpG ODN and DNA vaccine indicated the presence of an antigen-specific CMI response. Therefore this study confirms that Engerix B + CpG ODN and DNA immunization can influence the immune activation in chimpanzees to include antigen-specific CMI responses and that these strategies do possess the potential for therapeutic application in chronic HBV infection. Also these data represent one of the few demonstrations in which antigen-specific CTLs were generated using a protein-based vaccine. Unlike the mouse study, the chimpanzee study indicated that, in a large primate, the Engerix B + CpG ODN combination was more efficient that the DNA vaccine at generating an HBV-specific immune response. This was probably related to the level of optimization for each vaccine strategy. The CpG ODN combination vaccine was designed on top of a pre-existing HBV vaccine and the ODN sequence had been optimized for use in primates by screening on primate cells. Therefore, the inclusion of CpG ODN simply made it more efficient at inducing an immune response. The DNA vaccine, however, still remains to be efficiently optimized for delivery to higher primates.

4.6. Future work

The data presented in this work supports the merit of further investigation in to the use of protein/alum/CpG ODN and DNA vaccine strategies for the prevention and treatment of HBV infection.

HBsAg transgenic mouse studies

It was clear that the DNA vaccine and the combination of protein + CpG ODN were capable of negatively regulating HBsAg transgene expression in the Tg mouse. However, it would be interesting to undertake a closer evaluation of the characteristics of immune cells involved in this regulation. Although IFNγ production is crucial in mediating this effect, it is not completely clear what are the relative contributions of different IFNγ producing cells, such as CD4+ and CD8+ T cells, NK T cells and NK cells.

Further, this study focused on the generation of HBsAg-specific immune responses, however, prior to progression to humans it will also be important to evaluate the potential for generating immune responses to other HBV proteins in mice that are transgenic for these proteins. Although it is well established that strong humoral responses to HBsAg are protective, it remains to be determined which HBV antigens will be most important in a therapeutic setting.

Relation between antigen expression and immune activation following DNA vaccination

The relation between immune activation and antigen expression following DNA immunization revealed that antigen expression following transfection predisposes for immune mediated destruction by a number of mechanisms. A better understanding of the early events following DNA immunization and the relative contribution on transfected versus non-transfected APCs, as well as transfected

non-APCs, to immune activation and expansion will help guide investigation into the optimization of DNA vaccine delivery.

Humans and non-human primates

The Engerix B + CpG ODN vaccine combination has already entered phase I clinical trial to evaluate the potential of CpG ODN as an effective vaccine adjuvant in humans. Preliminary results indicate that observations made in the chimpanzees, with respect to humoral responses, will extend to humans (Davis et al. unpublished data). Ongoing evaluations will determine the ability of CpG ODN adjuvanted vaccines to induce CMI in humans.

With respect to the DNA vaccine, future investigation should focus on the optimization of vaccine delivery, such as vaccine dose and target sites. The goal of these studies should center on the transfection efficiency of candidate vaccination protocols. Recently, DNA vaccine doses as high as 10 mg were given to adult chimpanzees to generate anti-hepatitis C immunity with promising results (242). In comparison to the 2 mg given twice in this study and doses/kg given in mouse studies, this suggests that there is a lot of room for the evaluation of the influence of dose on the efficacy of DNA vaccines. Also, the understanding that immune cell presence throughout the body varies in different tissues supports evaluation of different delivery sites to ensure more efficient transfection and immune activation. A better understanding of the early events following DNA immunization in mice will help in this endeavor.

Ultimately these strategies will have to be evaluated in clinical trials carried out on HBV chronic carriers to determine their full potential as therapeutic agents. These trials should be designed to evaluate these strategies alone and in combination with antiviral therapies such as nucleoside analogs. Although antiviral therapies on there own have not proven to be completely effective, they may provide a beneficial effect with respect to decreasing viral and antigen burden when combined with vaccine therapy.

Based on the current developmental phase that each vaccine strategy is in, it is clear that the Engerix B + CpG ODN combination is the most promising of the two in the short term. However it remains important to evaluate the full potential of DNA vaccines since their success would revolutionize vaccinology.

Apendix

JOURNAL OF VIROLOGY, July 2001, p. 6482–6491
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.14.6482–6491.2001

Vol. 75, No. 14

CpG Oligodeoxynucleotides with Hepatitis B Surface Antigen (HBsAg) for Vaccination in HBsAg-Transgenic Mice

F. MALANCHÈRE-BRÈS,[1] P. J. PAYETTE,[2] M. MANCINI,[1] P. TIOLLAIS,[1] H. L. DAVIS,[2] AND M.-L. MICHEL[1]*

Unité de Recombinaison et Expression Génétique, INSERM U.163, Institut Pasteur, 75724 Paris Cédex 15, France,[1] and Loeb Health Research Institute at the Ottawa Hospital, Ottawa, Ontario, Canada[2]

Received 14 December 2000/Accepted 24 April 2001

DNA motifs containing unmethylated CpG dinucleotides within the context of certain flanking sequences enhance both innate and antigen-specific immune responses, due in part to the enhanced production of Th1-type cytokines. Here we explored the ability of CpG-containing oligodeoxynucleotides combined with recombinant hepatitis B surface antigen (HBsAg) to induce Th1 responses in mice that are transgenic for this antigen and that represent a model for asymptomatic hepatitis B virus chronic carriers. This was compared to hepatitis B virus-specific DNA-mediated immunization, which we have previously shown to induce the clearance of the transgene expression product and the down-regulation of hepatitis B virus mRNA in this transgenic mouse lineage. In control nontransgenic C57BL/6 mice, three immunizations with HBsAg and CpG triggered the production of anti-HBs antibodies and of HBs-specific T cells that secrete gamma interferon but do not display any HBsAg-specific cytotoxic activity. In the HBsAg-transgenic mice, immunization with HBsAg and CpG oligodeoxynucleotides, but not with CpG alone, induced the clearance of HBsAg circulating in the sera, with a concomitant appearance of specific antibodies, and was able to regulate the hepatitis B virus mRNA constitutively expressed in the liver. Finally, adoptive transfer experiments with $CD8^+$ T cells primed in C57BL/6 mice with HBsAg and CpG oligodeoxynucleotide-based immunization show that these cells were able to partially control transgene expression in the liver and to clear the HBsAg from the sera of recipient transgenic mice without an antibody requirement. CpG oligodeoxynucleotides motifs combined with HBsAg could therefore represent a potential therapeutic approach with which to treat chronically infected patients.

Hepatitis B virus (HBV) causes a common infectious disease, and there are an estimated 350 million chronic HBV carriers worldwide (29). Patients with chronic hepatitis B are at high risk of developing liver cirrhosis, and this is associated with a higher rate of mortality due to the development of hepatocellular carcinoma or noncarcinomatous complications of cirrhosis (20, 21).

Currently, the only therapy for chronic hepatitis that has a lasting beneficial effect is systemic treatment with alpha interferon (IFN-α), but a sustained response is achieved in only one-third of patients with chronic hepatitis B (21). Nucleoside analogues such as lamivudine provide a therapeutic alternative leading to a rapid decrease in serum HBV DNA levels and to histopathological improvement of liver disease. However, cessation of treatment usually leads to a rapid relapse of disease, and long-term treatment often results in the selection of resistant viral variants (27). These outcomes emphasize the need for novel therapeutic approaches. Although the pathogenesis of chronic liver disease is not well understood, there is a consensus that liver damage is immune mediated. Specific immunotherapeutic strategies have been proposed as possible alternatives to the use of IFN or antiviral drugs to enhance or to broaden the defective T-cell responses in chronically infected patients. Among these, specific vaccine therapies with either currently available recombinant anti-hepatitis B vaccines (9, 40), a lipopeptide-based T-cell vaccine (53), or newly developed genetic vaccines (31, 33, 42) have been studied recently with animal models or in human clinical trials (19, 40).

As an animal model for asymptomatic carriers infected at birth, we have used mice that are transgenic (Tg) for hepatitis B surface antigen (HBsAg) (1, 16). In this model, we have previously shown that HBsAg-specific T- and B-cell responses induced after DNA-based immunization are able to mediate antigen clearance in the sera and down-regulation of transgene expression in the liver (33, 34). The Th1 bias of the immune response induced following intramuscular (i.m.) injection of DNA is mostly attributed to immunostimulatory CpG motifs present in the plasmid (44). Thus, we ask whether synthetic CpG-containing oligodeoxynucleotides (ODN) could efficiently replace DNA adjuvanticity for HBsAg immunization in this Tg mouse lineage.

Unmethylated cytosine-guanine dinucleotides within the context of certain flanking sequences (CpG motifs), as originally identified in bacterial DNA, have diverse stimulatory effects on the innate and adaptive immune systems. Several of these effects contribute to the strong Th1-type adjuvant activity for antigen-specific responses. For example, CpG DNA triggers most (>95%) B cells to proliferate, secrete immunoglobulin (Ig) and cytokines, and be protected from apoptosis (24, 26, 57), all of which contribute to a stronger humoral response. CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete various Th1 cytokines (18, 24), which in turn induces T and NK cells to secrete additional cytokines (2, 4, 10, 24, 56, 57). Overall, CpG induces a strong Th1-like pattern of cytokine production dominated by inter-

* Corresponding author. Mailing address: Unité de Recombinaison et Expression Génétique, INSERM U.163, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cédex 15, France. Phone: 33(1 45 68 88 49. Fax: 33(1 45 68 89 43. E-mail: malcum@pasteur.fr.

leukin-12 (IL-12) and IFN-γ, with little secretion of Th2 cytokines (24), and these cytokines can provide additional T-cell help for both humoral and cell-mediated immune responses. CpG ODN have been shown to be effective Th1-type vaccine adjuvants in animals with a variety of antigens. For example, mice immunized by i.m. injection of antigen with CpG ODN have strong cytotoxic T lymphocytes (CTL) and predominantly IgG2a antibodies, also indicative of a Th1-type response (8, 12, 30, 43, 55). Since such Th1-type immune responses are thought to be necessary for clearance of HBV infection (3, 17, 23, 39), it is possible that CpG ODN with recombinant HBsAg may be an effective therapeutic vaccine for the treatment of patients chronically infected with HBV. Here, we show that immunization with HBsAg combined with CpG ODN resulted in clearance of the HBsAg from the sera, induction of specific antibodies, and partial down-regulation of HBV mRNA in the livers of HBsAg-Tg mice.

MATERIALS AND METHODS

Antigens used for immunization. The pCMV-S2.S DNA (37), which served as a positive control, expressed the S and pre-S2 region of the HBV envelope gene (*ayw* subtype) under the control of the cytomegalovirus (CMV) immediate-early gene promoter. The plasmid DNA used for gene transfer was purified with anion-exchange chromatography columns (Qiagen GmbH, Hilden, Germany), redissolved in endotoxin-free phosphate-buffered saline (PBS) (Sigma, St. Louis, Mo.) at 1 mg/ml, and stored at or below −20°C. The protein vaccine contained recombinant surface proteins of HBV (small and middle proteins encoded by S and pre-S2 genes) of the *ayw* subtype, which was produced in CHO cells (PMC, Val de Reuil, France) (36) and is referred to hereafter as HBsAg. Unless otherwise indicated, the antigen was used in soluble form, without any adjuvant, at a final concentration of 1 mg/ml. HBsAg was combined with CpG ODN (sequence 1826, 5′ TCCATGACGTTCCTGACGTT 3′ [optimal murine CpG motifs are underlined]) or non-CpG control ODN (sequence 1982, 5′ TCCAGGACTTCTCTCAGGTT 3′) (12). The ODN, which were provided by the Coley Pharmaceutical Group (Wellesley, Mass.), were synthesized with a nuclease-resistant phosphorothioate backbone.

Peptides. The eight-mer synthetic K^b-binding S 371–378 peptide ILSPFLPL was synthesized by Genosys Biotechnologies (Cambridge, United Kingdom), and the 13-mer 126–138 peptide RGLYFPAGGSSSG was obtained from Neosystem (Strasbourg, France). The numbering of the amino acid sequence of peptides starts from the first methionine of the HBV *ayw* subtype pre-S domain. These peptides were used for the CTL and enzyme-linked immunospot (ELISPOT) assays.

In vitro splenocyte stimulation assays. Spleens from 10- to 12-week-old naive C57BL/6 mice (Charles River, Wilmington, Mass.) were recovered under sterile conditions, and single-cell suspensions were prepared in RPMI 1640 (Life Technologies, Gaithersburg, Md.) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2% (vol/vol) heat-inactivated normal mouse serum (NMS) (Cedarlane, Hornby, Canada). The splenocytes were plated at 5×10^6/ml for the cytokine assays (100 μl/well) in triplicate in 96-well round-bottom polystyrene plates (Becton Dickinson Labware, Franklin Lakes, N.J.). CpG ODN 1826 and non-CpG control ODN 1982 were suspended in complete RPMI 1640 and plated at 100 μl/well to final concentrations of 10, 3, 1, and 0.3 μg/ml for the cytokine evaluation. For comparison, genomic *Escherichia coli* DNA, as a source of bacterial DNA, was suspended in RPMI 1640 without sera and plated at 100 μl/well to final concentrations of 10 and 30 μg/ml. The splenocytes were incubated for 96 h at 37°C and 5% CO_2. For cytokine evaluation, four identical assays were set up, and culture supernatants were harvested and stored at −80°C following 6, 24, 48, and 72 h of incubation at 37°C and 5% CO_2. The levels of tumor necrosis factor alpha (TNF-α), IL-12, IL-6, and IFN-γ were determined by using murine-specific OPTEIA enzyme-linked immunosorbent assay (ELISA) sets (Pharmingen, Mississauga, Ontario, Canada).

Immunization of mice. Normal C57BL/6 and HBsAg-Tg mice (1) were kept under standard pathogen-free conditions in the animal facility of the Pasteur Institute. The HBV envelope Tg mouse lineage E36 was initially produced on a C57BL/6 × SJL/J background and was then backcrossed against C57BL/6 (H-2^b) at least 24 times before use. The transgene in these mice consists of a copy of the HBV genome with the core gene deleted. The transgene is expressed exclusively in the liver, and large amounts of HBsAg particles are secreted in mouse serum (16, 33). C57BL/6 Tg or non-Tg female mice, 5 to 7 weeks old, were immunized two or three times at monthly intervals by i.m. injection bilaterally into the tibialis anterior (TA) muscle with recombinant HBsAg (2 μg) alone or combined with CpG or non-CpG ODN (120 μg) in a total volume of 50 μl per leg. As a positive control, other Tg and non-Tg mice were injected on a single occasion with 100 μg of recombinant plasmid DNA directly into regenerating TA muscles as previously described (32). We have shown this to induce strong HBsAg-specific immunity and to bring about control of transgene expression in the Tg mice (33).

Serologic test. At various times before and after DNA injection, blood was collected from mice by retroorbital puncture with heparinized glass pipettes, and sera recovered by centrifugation were assayed for anti-HBs and anti-preS2 antibodies by specific ELISA. Purified recombinant particles containing HBV small S protein (1 μg/ml) or pre-S2 (120–145) synthetic peptide (1 μg/ml) were used as the solid phase. After blocking with PBST (PBS containing 0.1% Tween 20) supplemented with 10% fetal calf serum, serial dilutions of sera were added. After extensive washing, the bound antibodies were detected with antimouse Ig (total IgG) labeled with horseradish peroxidase (Amersham, Little Chalfont, United Kingdom). Antibody titers were determined by the serial end-point dilution method. Mouse sera were tested individually, and titers were the mean of at least three determinations. Serum dilutions below 1/100 were considered negative. The sera from DNA-immunized HBsAg-Tg mice were also used for detection of HBsAg with a commercial ELISA kit (Monolisa AgHBs; Bio-Rad, Marnes la Coquette, France). Anti-HBs and HBsAg titers were expressed as group geometric means ± standard errors of the mean of individual animal values, which represent the average of duplicate or triplicate assays. The significance of differences between values was assessed with a Mann-Whitney test; *P* values lower than 0.05 were considered significant.

CTL activity assay. Groups of four C57BL/6 mice were immunized with HBsAg combined with CpG or non-CpG ODN or with pCMV-S2.S DNA vector. Spleens were removed from immunized mice 2 weeks after the last injection of HBsAg and ODN or 4 weeks after the DNA injection. Single-cell suspensions were prepared. Cells (10^7 cells/well) were suspended in 2 ml of α minimum essential medium (α-MEM) supplemented with 10 mM HEPES buffer, 1 mM sodium pyruvate, nonessential amino acids, 0.05 mM β-mercaptoethanol, antibiotics, glutamine, and 10% fetal calf serum (Myoclone; Gibco BRL; Cergy Pontoise, France) in 24-well plates. Responder spleen cells were cocultured with 10^6 irradiated autologous transfected cells expressing the small envelope protein (RBL5.S) or with RBL5 cells pretreated with HBsAg particles. After 5 days in culture, half of the medium was replaced with fresh medium, and the cells were used as effectors in a standard chromium release assay performed 2 or 3 days later. For pulsing, 10^6 RBL5 cells were incubated with 10 μg of HBsAg in 500 μl of complete α-MEM for 2 h at 37°C with 5% CO_2. After two washes in α-MEM medium, cells were irradiated at 200 Gy and then used as stimulator or target cells in the CTL activity assay (45, 46). Targets were autologous transfectant cells (RBL5.S), HBsAg-pulsed or peptide (HBs 371–378)-pulsed RBL5 cells, or unpulsed RBL5 cells. Targets were labeled with ^{51}Cr (3.7 MBq/10^6 cells; Amersham). After a 4-h incubation at 37°C, 50 μl of supernatant was removed from each well and counted on a beta counter as described elsewhere (34). The percentage of specific release was calculated as [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Total release was measured by resuspending target cells in lysis buffer. Spontaneous release was obtained from targets incubated with medium alone and is usually less than 15% of the total release.

ELISPOT assay. The number of splenic IFN-γ-secreting cells was determined by using a modification of the Czerkinsky ELISPOT technique (11). In brief, flat-bottom nitrocellulose ELISA plates (Multiscreen; Millipore, Molsheim, France) were coated overnight with rat anti-mouse IFN-γ (Pharmingen, San Diego, Calif.) and thereafter saturated for 2 h at 37°C with RPMI 1640 containing 10% fetal calf serum (complete RPMI medium). Cells were suspended in complete RPMI medium, transferred onto coated plates (10^6 cells/well), and incubated for 42 h at 37°C in 5% CO_2 with stimulator peptides or antigen. Two different peptides were used at the concentration of 3 μg/ml: the 13-mer major histocompatibility complex (MHC) class II binding 126–138 peptide (34) and the 8-mer H-2K^b-binding HBs 371–378 peptide (47). The cells were removed by flicking the plates and then lysed with water. After being washed with PBS–0.05% Tween 20, biotinylated rat anti-mouse IFN-γ antibody (Pharmingen) was added to each well. Following incubation and subsequent washing, the plates were incubated with streptavidin-alkaline phosphatase conjugate (Boehringer-Mannheim, Germany). Next, a 2.3 mM solution of 5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, Wis.) diluted in alkaline buffer solution was added. When spots were visible, the color reaction was stopped by rinsing the plates with distilled water. Then, after drying, the number of IFN-γ-secreting

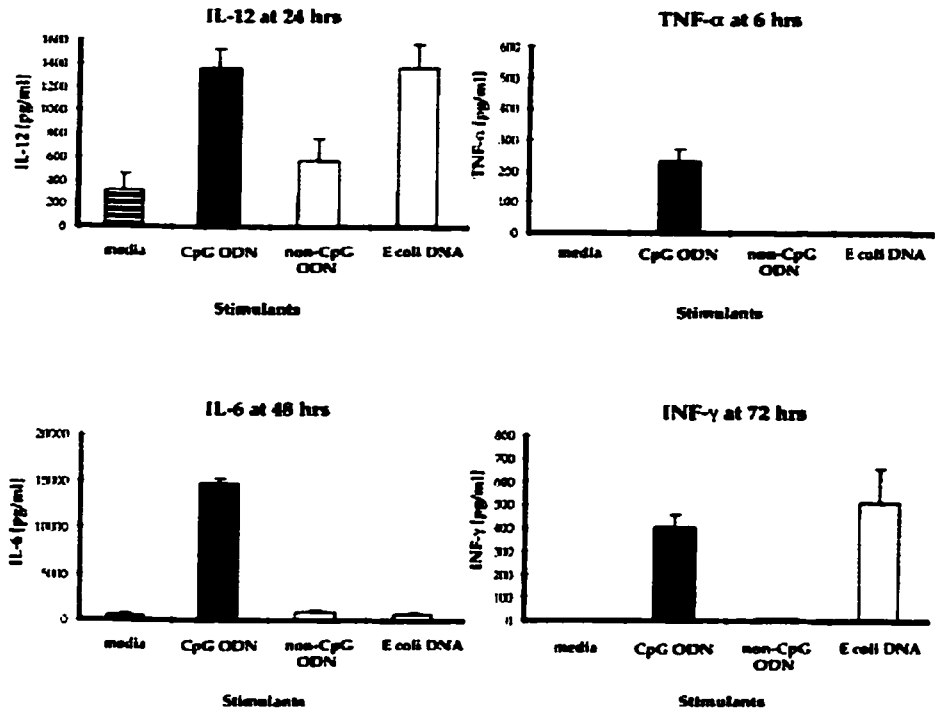


FIG. 1. In vitro cytokine production by splenocytes from naive C57BL/6 mice stimulated with CpG (solid bars) or non-CpG ODN (open bars) at 3 μg/ml or *E. coli* DNA (shaded bars) at 30 μg/ml. Supernatants were collected at different time points after stimulation, and cytokines were quantified by ELISA. The results show the means of quadruplicate experiments.

nine spots was counted. Each cell population was titrated in triplicate, but data were derived only from wells with more than 10 spots.

CD8⁺ T-lymphocyte subset fractionation and adoptive transfer. The CD8⁺ T-cell subpopulation was isolated from the total spleen cells by negative selection by magnetic cell sorting (MACS; Miltenyi Biotec, Paris, France). The purity of the CD8⁺ T cell was confirmed by cytofluorometry analysis with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, Calif.) with Cell Quest software and following staining with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD8 and anti-CD4 monoclonal antibodies (Pharmingen, San Diego, Calif.). The percentage of undesirable CD4⁺ T cells in the population was <0.6%. The remaining leukocytes were pooled according to the type of immunization and then counted and resuspended in 200 μl of endotoxin-free PBS. CD8⁺ T cells (10^6 to 10^7) were injected into the retroorbital cavities of recipient mice that had been sublethally irradiated (5 Gy) before transfer.

Northern blot analysis. Total RNA of liver was extracted from mechanically pulverized frozen tissue with RNA-PLUS (Biogreb Systems, Montreuil-sous-Bois, France). The RNA (40 μg) was fractionated on 1% formaldehyde–agarose gels and blotted onto nylon membranes, which were then hybridized with ^{32}P-DNA probes synthesized from a 2.4-kb *Bgl*II HBV DNA fragment or from a 0.2-kb *Pst*I cDNA fragment of the murine 18S rRNA gene (Valbiotech, France) by using the Rediprime DNA labeling system (Amersham). Quantification of HBV mRNA was performed with a PhosphorImager.

Statistical test. The statistical test used to calculate *P* values for differences in mRNA levels was the nonparametric Mann-Whitney test. It was used with Stat View 4.5 software (Abacus Concepts, Berkeley, Calif.). *P* values < 0.05 were considered significant.

RESULTS

In vitro effect of ODN on cytokine production by naive C57BL/6 splenocytes. The immunostimulatory effects of CpG ODN were evaluated with spleen cells derived from nonimmune C57BL/6 mice. Pooled splenocytes from five naive mice were cultured in vitro either alone or with CpG ODN, non-CpG control ODN, or *E. coli* DNA. The CpG ODN clearly showed a superior ability to induce a non-antigen-specific lymphoproliferative response when compared to medium, the control ODN, or *E. coli* DNA (data not shown). With respect to cytokine production, the CpG ODN, but not the control ODN, induced significant levels of TNF-α, IL-6, and IFN-γ (Fig. 1). Comparable amounts of IFN-γ and IL-12 were induced from splenocytes after stimulation with CpG ODN and *E. coli* DNA. Presumably because of the optimized CpG motifs and a nuclease-resistant phosphorothioate backbone, the CpG ODN was superior to both control ODN and *E. coli* DNA at inducing detectable TNF-α and IL-6 production.

Effect of ODN on humoral response in C57BL/6 mice immunized with HBsAg. Groups of six mice were immunized i.m. once with pCMV-S2.S DNA or three times with HBsAg alone

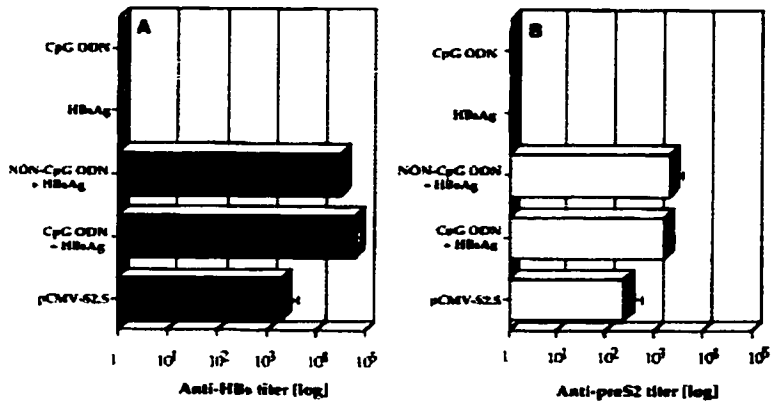


FIG. 2. Anti-hepatitis B Ig-specific responses. Groups of six C57BL/6 mice were immunized once with pCMV-S2.S (100 μg) or three times at monthly intervals with CpG ODN alone (120 μg), HBsAg alone (2 μg), or HBsAg combined with CpG or non-CpG control ODN. Antibodies specific for HBsAg (A) or the pre-S2 domain of HBV middle protein (B) were investigated by ELISA 3 weeks after the last injection. Antibody titers are expressed as a serial end point dilutions.

or combined with CpG or non-CpG control ODN, with other controls receiving CpG ODN alone. Antibodies specific for HBsAg (Fig. 2A) or the pre-S2 domain of HBV middle protein (Fig. 2B) were detected by ELISA 3 weeks after the last injection. Both ODN combined with HBsAg induced a significant production of anti-HBs and anti-pre-S2 antibodies in C57BL/6 mice (Fig. 2). In contrast, HBsAg alone was not able to induce specific antibodies. As expected, no anti-HBs antibodies were detected in mice receiving CpG ODN alone. Interestingly, the antibody titers were comparable ($P < 0.165$) in the sera of mice receiving HBsAg with either CpG ODN or non-CpG control ODN. As previously shown (37), a single injection of pCMV-S2.S DNA was sufficient to induce anti-HBs and anti-pre-S2 antibodies. Anti-HBs antibody titers induced after injection of HBsAg combined with CpG and non-CpG ODN were 30- and 16-fold higher, respectively, than those obtained after DNA immunization. Similarly, titers of anti-pre-S2 antibodies were six- and eightfold higher in mice receiving HBsAg with CpG and non-CpG ODN, respectively. However, it should be noted that these antibodies were induced after three injections of HBsAg with ODN, whereas the DNA was given only once.

The CpG ODN coinjected with the HBsAg in the C57BL/6 mice induced a production of high levels of anti-HBs and anti-pre-S2-specific antibodies. This specific antibody production was concomitant with a significant increase in the IgG2b titer in the C57BL/6 mice (data not shown). In this strain, IgG2a antibodies are not detectable due to a missing gene (22). The adjuvant effect of control ODN was unexpected, since this had not been seen before in BALB/c mice (12). This could be due in part to the ODN dose, which was 10 times higher than that used in previous studies with BALB/c mice. In addition, some adjuvant properties of the nuclease-resistant phosphorothioate backbone had previously been observed with mucosal administration (35).

In vivo adjuvant effect of CpG ODN on the cellular immune response in C57BL/6 mice immunized with HBsAg. C57BL/6 mice were immunized twice (1 month apart) with HBsAg (2 μg) together with the CpG ODN (120 μg). Spleen cells were taken 2 weeks after the second injection. As a positive control, C57BL/6 mice were immunized with pCMV-S2.S (100 μg) as already described (32) and sacrificed 4 weeks after the single injection. Primed T splenocytes recovered from C57BL/6 mice were restimulated in vitro with either irradiated HBsAg-pulsed RBL5 cells (Fig. 3 B and D, exogenous antigenic stimulation) or irradiated HBs-expressing RBL5/S transfected cells (Fig. 3A and C, endogenous antigenic stimulation). The targets were autologous transfectant cells expressing or not expressing the HBV small envelope protein or cells pulsed with HBV peptide (HBs 371–378) or with HBsAg. The results show that immunization with HBsAg with CpG ODN (Fig. 3A and B) did not induce HBsAg-specific CTL irrespective of whether in vitro endogenous or exogenous restimulation was used. In contrast, DNA-based immunization with HBsAg-encoding plasmid pCMV-S2.S (Fig. 3C and D) triggered the emergence of HBsAg-specific CTL after in vitro restimulation with endogenously processed antigen (Fig. 3C). Likewise, after stimulation with exogenously processed antigen, the DNA vaccination generated CTL specific for target cells pulsed with HBsAg or with the peptide HBs 371–378 (Fig. 3D), but not for peptides presented by RBL5/S transfected cells. HBsAg-specific CTL were not observed in C57BL/6 mice immunized with HBsAg alone or in control mice administered CpG ODN without antigen (data not shown).

Effect of CpG ODN on T-cell cytokine secretion in C57BL/6 mice immunized with HBsAg. We performed an anti-IFN-γ ELISPOT assay to evaluate the frequency of HBsAg-specific T cells in the spleens of C57BL/6 mice after immunization with HBsAg plus CpG ODN, with an HBsAg-expressing DNA vac-

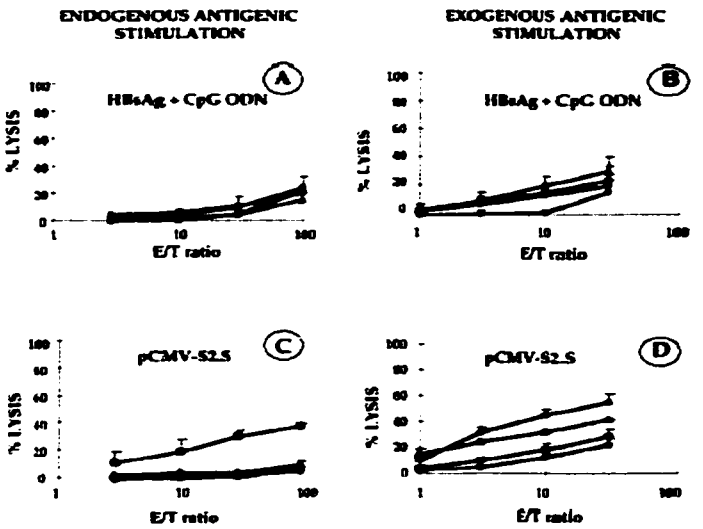


FIG. 3. CTL responses of C57BL/6 mice immunized once with pCMV-S2.S (100 μg) or twice, 1 month apart, with HBsAg (2 μg) combined with CpG ODN (120 μg). Splenocytes harvested, respectively, 1 and 2 weeks postimmunization were stimulated in vitro for 5 days with irradiated HBsAg-pulsed RBL5 cells (exogenous stimulation) or with irradiated HBs-expressing RBL5/S transfectant cells (endogenous stimulation). The target cells used were RBL5 cells (●), RBL5/S transfectant cells (■), HBsAg-pulsed RBL5 cells (▲), or peptide 371–378-pulsed RBL5 cells (□). The percentage of specific lysis was calculated as [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(total ^{51}Cr release − spontaneous ^{51}Cr release)] × 100. Results represent the mean for four mice.

tor, or with HBsAg or CpG ODN alone. The number of epitope-specific T cells producing IFN-γ was measured with the HBs 371–378 K^b-binding peptide (Fig. 4A) or the HBs 126–138 T helper peptide (Fig. 4B) as stimulatory peptides. The results show that, compared to the immunization with HBsAg alone, immunization with HBsAg plus CpG ODN or with pCMV-S2.S DNA was able to activate, respectively, 6 or 12 times more HBs-specific IFN-γ-secreting $CD8^+$ T cells (Fig. 4A). In addition, stimulation with the T helper peptide HBs 126–139 induced IFN-γ production by spleen cells from mice immunized with pCMV-S2.S DNA, HBsAg plus CpG ODN, or HBsAg (Fig. 4B), but the frequency of such IFN-γ-secreting $CD4^+$ T cells was significantly higher in mice immunized with HBsAg plus CpG ODN than in mice receiving HBsAg alone. In DNA-immunized control mice, the number of INF-γ-secreting T cells was 10 times higher than that in mice immunized with HBsAg alone.

DNA or CpG ODN immunization of HBsAg-Tg mice induced the clearance of circulating HBsAg and the concomitant appearance of anti-HBs and anti-pre-S2 antibodies. We have previously described how a single injection of pCMV-S2.S DNA that encodes HBsAg, but not an irrelevant DNA, into HBsAg-Tg mice induced a persistent decrease in circulating HBsAg particles and a concomitant appearance of serum anti-HBs antibodies, which were maintained over time (33). Figure 5A shows that three injections of HBsAg combined with immunostimulatory CpG ODN were sufficient to clear HBsAg from the sera of HBsAg-Tg mice to a similar degree to clearance with the DNA vaccine. In contrast, three injections of CpG ODN alone, HBsAg alone, or HBsAg combined with non-CpG control ODN did not result in major changes in HBsAg levels (Fig. 5A).

Specific antibodies were detectable at the time of HBsAg decrease in the sera of Tg mice. Anti-HBs (Fig. 5B) and anti-pre-S2 (Fig. 5C) antibodies appeared in the sera of pCMV-S2.S-immunized Tg mice by 4 and 8 weeks, respectively, after a single injection of DNA, and these continued to increase over time. Using HBsAg combined with CpG ODN, three injections were required to obtain comparable anti-preS2 antibody titers (Fig. 5C) and anti-HBs titers that were 50-fold greater at the peak of the response than those induced by the DNA vaccine (Fig. 5B). As with the DNA vaccine, injections of HBsAg plus CpG ODN in the HBsAg-Tg mice resulted in the clearance of serum HBsAg and the appearance of detectable specific antibodies.

In contrast, compared to the C57BL/6 non-Tg mice (Fig. 2), injections of HBsAg combined with non-CpG control ODN did not induce any detectable anti-pre-S2 antibodies and only very low levels of anti-HBs antibodies (in two out of nine mice), which failed to trigger clearance of HBsAg.

Combination of CPG ODN with HBsAg was able to regulate the HBV mRNA expression in the livers of HBsAg Tg mice. Northern blot analysis of mRNA from livers of Tg mice sacrificed at the time of HBsAg clearance (15 weeks after immunization) was performed, and HBV mRNA was quantified with a PhosphorImager after correction for mRNA loading and

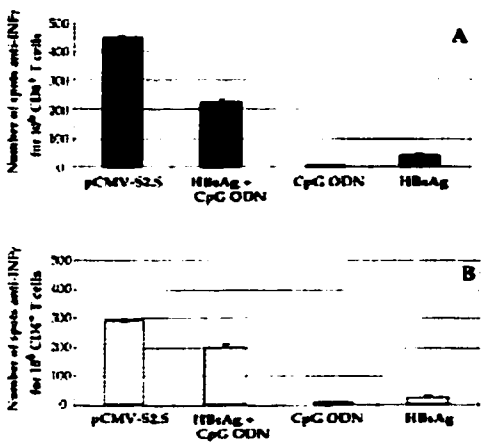


FIG. 4. INF-γ production by ELISPOT. Groups of four C57BL/6 mice were immunized (i.m.) twice at monthly intervals with recombinant HBsAg (2 μg) alone or combined with CpG ODN (120 μg), with CpG ODN alone (120 μg), or once with pCMV-S2.S (100 μg). Each bar represents the mean of triplicate values for spot-forming cells following stimulation of splenocytes with peptide 371–378 for 10^6 $CD8^+$ T cells (A) or with peptide 126–138 for 10^6 $CD4^+$ T cells (B).

variations in transfer efficiency as assessed by 18S rRNA expression. We have previously shown that the immune response induced after a single injection of pCMV-S2.S DNA into HBsAg-Tg mice reduced the level of HBVmRNA in the liver, in some cases to undetectable levels (33). In the present study, both the DNA vaccine and HBsAg plus CpG ODN reduced the mean HBV mRNA level in the livers of mice to a similar degree ($P < 0.30$) (Fig. 6). The mean HBV mRNA levels in the livers of mice receiving HBsAg plus non-CpG ODN or HBsAg alone were not significantly different ($P < 0.60$). Untreated age- and sex-matched Tg mice displayed a range of HBV mRNA levels identical to that of mice receiving HBsAg alone ($P < 0.90$). In contrast, the mean HBV mRNA levels in the livers of mice receiving HBsAg plus non-CpG ODN or HBsAg alone were significantly higher than in mice treated with HBsAg plus CpG ODN ($P < 0.02$ and $P < 0.025$, respectively) and appeared not to be reduced. These data showed that three injections of HBsAg combined with CpG ODN were as efficient as a single injection of pCMV-S2.S DNA at reducing HBV mRNA in the livers of HBsAg-Tg mice and that the effect was CpG dependent.

The $CD8^+$ T cells primed by HBsAg plus CpG ODN immunization induced the clearance of HBsAg in the sera and the control of HBV mRNA in the livers of HBsAg-Tg mice. Because $CD8^+$ T cells detected after immunization with HBsAg plus CpG ODN did not display any detectable cytolytic activity in vitro, we sought to determine whether they might nevertheless be responsible for the control of HBV mRNA expression observed in vaccinated Tg mice. We thus performed adoptive transfer experiments. As a positive control, we used $CD4^+$-depleted spleen cells from DNA-immunized mice, which have been shown previously to down-regulate transgene expression in the livers of HBsAg-Tg mice. Indeed, HBsAg was completely cleared from the sera of all five recipient mice by 50 days following transfer of pCMV-S2.S-primed $CD4^+$-depleted T cells, whereas it was cleared in only three mice and reduced in the other two after transfer of HBsAg plus CpG ODN-primed cells (Fig. 7A). No anti-HBs antibodies were detected in the sera of the recipient mice 50 days after adoptive transfer. Northern blot analysis of RNA prepared from the livers of the recipient Tg mice shows that HBV mRNA was still clearly detectable in the livers of the two of five mice receiving HBsAg plus CpG ODN-primed T cells that failed to clear HBsAg and was almost undetectable in the other three mice, as well as the five of five mice receiving pCMV-S2.S-primed T cells (Fig. 7B).

These results show that $CD8^+$ T cells primed by HBsAg adjuvanted with CpG ODN were able to down-regulate HBV mRNA in the livers of HBsAg-Tg mice in the absence of detectable in vitro cytolytic activity, but to a somewhat lesser extent than those primed by the DNA vaccine.

DISCUSSION

In this study, we have evaluated the adjuvant effect of CpG ODN on the immune response to HBsAg in either naive or HBsAg-Tg mice. With this mouse model of HBV chronic carriers, we have previously shown that vaccination with DNA plasmid encoding HBsAg can break nonresponsiveness to this antigen and resulted in the clearance of HBsAg from the sera and the down-regulation of HBV mRNA in the liver. However, the efficiency of the immune response obtained after DNA-based immunization has been described as being due in part to the presence of immunostimulatory CpG motifs contained in the plasmid backbone (44). These sequences are thought to be responsible for the Th1 response observed after genetic immunization, whereas protein vaccines induce mostly Th2 responses. Thus, CpG ODN could represent an alternative way of modulating the immune response to classical recombinant antigens towards a Th1 profile.

The CpG and the control ODN coinjected with the HBsAg in C57BL/6 mice induced a strong humoral response with high production of anti-HBs and anti-pre-S2-specific antibodies. However, in HBsAg-Tg mice, only the ODN containing consensus CpG motifs were able to induce antibodies, which efficiently clear the transgene-encoded HBsAg from mice sera. Antibody response in non-Tg C57BL/6 mice is dependent on T helper cells specific for epitopes present in both S and pre-S2 domains (38). In HBeAg-Tg mice, it has been shown that T helper cells survive deletion or anergy in the presence of circulating antigen by virtue of low avidity, but they are nevertheless capable of being activated (5). Thus, in our HBsAg-Tg mice, activation of such T helper cells may require a strong activation through CpG ODN to provide help for antibody production. Alternatively, CpG DNA has been shown to directly activate B cells to proliferate in a T-cell-independent manner, and this effect is synergistic with B-cell activation through the antigen receptor (25).

The CpG ODN but not the control ODN has a Th1-type effect and allows IFN-γ secretion from splenocytes stimulated either in vitro or in vivo after immunization. In vitro, after stimulation with the CpG ODN, the C57BL/6 splenocytes pro-

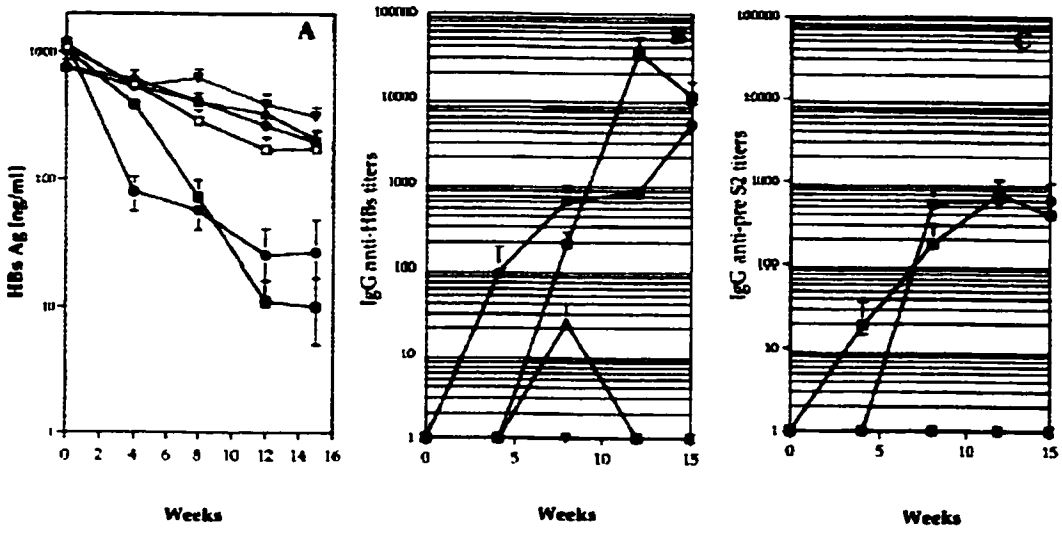


FIG. 5. Immunization of HBsAg-Tg mice. Groups of Tg mice were injected with PBS instead of DNA (nonimmunized, ▯ mice [○]) or immunized once with pCMV-S2.S (12 mice; ●) or three times at monthly intervals with CpG ODN alone (5 mice; □) or with either HBsAg alone (6 mice; ▼) or combined with CpG (12 mice; ■) or the non-CpG control (9 mice; ▲) ODN as in Fig. 2. Mice were bled at weekly intervals, and the sera were analyzed for HBsAg expressed as nanograms per milliliter (A) and for anti-HBs-specific (B) and anti-pre-S2-specific (C) antibodies (expressed as serial end point dilutions). Each point represents the mean titer for the group, and error bars represent the standard error.

duced Th1 cytokines such as IL-12, INF-γ, and TNF-α (Fig. 1). This could favor a Th1 environment for the development of antigen-specific T cells after immunization. In addition, IL-6, a cytokine known to activate B cells, is produced by CpG ODN-activated splenocytes. In vivo, coinjection of CpG ODN with HBsAg has a clear adjuvant effect in inducing HBsAg-specific $CD4^+$ and $CD8^+$ T cells that secrete IFN-γ (Fig. 4) as well as B cells, which produce anti-HBs antibodies (Fig. 2).

Thus, in C57BL/6 mice, the HBsAg plus CpG ODN immunization mimicked the DNA vaccine with respect to humoral response and to IFN-γ production by T cells. However, these results are different from those reported for BALB/c mice, in which HBsAg plus CpG ODN induced a potent CTL response as well (12). After a single pCMV-S2.S DNA injection, the immunized C57BL/6 mice produced a strong HBV-specific CTL response. As shown by our model of i.m. immunization with pCMV-S2.S, we have some experimental evidence that DNA vaccination results in the in vivo production of HBsAg particles (13). It has been demonstrated that HBsAg can be processed by an alternative pathway for peptide presentation by MHC class I molecules (46). This results in peptides different from those derived from the classical endogenous pathway (47). Therefore, induction of CTL specific for the peptide HBs 371–378 that result only from the exogenous pathway of degradation indicates that the antigen produced after DNA-based immunization has been endocytosed or captured, processed in the alternative pathway, and presented as peptides in association with MHC class I molecules. In contrast after immunization of C57BL/6 mice with HBsAg and CpG ODN, we did not detect a cytotoxic response. To be sure that the lack of CTL response in the C57BL/6 mice was not due to technical difficulties, we stimulated splenocytes in vitro under the following different experimental conditions: (i) with autologous RBL5/S transfectant cells (46) expressing the small envelope protein (endogenous condition) (45), (ii) with HBsAg-pulsed RBL5 cells (exogenous condition), and (iii) with unpulsed RBL5 cells as a control. None of these experimental strategies has been able to elicit in vitro a cytotoxic activity of lymphocytes primed in vivo with HBsAg plus CpG ODN. Thus the $CD8^+$ T cells induced by HBsAg plus CpG ODN immunization secreted IFN-γ, but did not display cytotoxic activity in vitro (52). Nevertheless, both DNA vaccine and HBsAg plus CpG ODN broke the nonresponsiveness to HBsAg in Tg mice by inducing clearance of the transgene expression product and control of HBV mRNA in the liver. After direct immunization of HBsAg-Tg mice, the clearance of HBsAg is mediated in part by the high-titer anti-HBs antibody production triggered by immunization, but also by the regulation of transgene expression at the mRNA level. In this Tg mouse model, we have previously shown that antibodies alone are not sufficient to achieve a persistent clearance of the antigen and that IFN-γ-secreting T cells are required (33, 34). In addition, overcoming nonresponsiveness to HBsAg in Tg mice immunized with HBsAg plus CpG ODN seems to be mediated by $CD8^+$ T cells, as demonstrated by experiments involving adoptive transfer of $CD4^+$-depleted splenocytes into Tg mice. In these adoptive

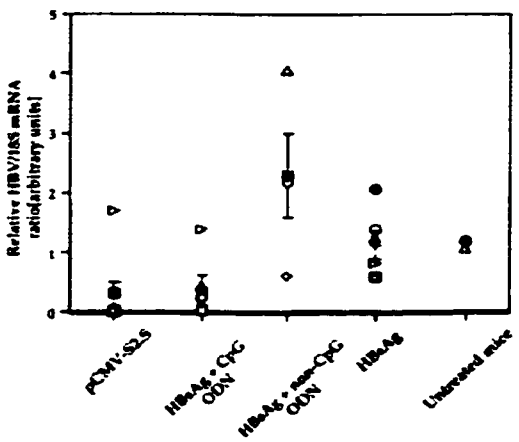


FIG. 6. Analysis of HBV mRNA content in the livers of Tg mice was performed by Northern blotting and quantification with PhosphorImager. Livers from Tg mice were taken 15 weeks after immunizations with pCMV-S2.S or with HBsAg alone or combined with CpG or non-CpG-control ODN as in Fig. 5. The HBV/18S mRNA ratios from untreated mice are shown as a control. DNA probes specific for HBV and 18S RNA were used, and the HBV/18S mRNA ratio is expressed in arbitrary units. Values representing individual data, the mean, and the standard error are shown.

transfer experiments, although B cells were transferred with $CD8^+$ T cells, no specific antibodies were detected at the time of HBsAg elimination, ruling out a role for an antibody-mediated clearance. Recent experiments with adoptive transfer of $CD8^+$-depleted splenocytes into Tg mice resulted in the clearance of HBsAg from the sera as well. Moreover, detection of CTL specific for the HBs 371–378 Kb-binding peptide after peptide stimulation of splenocytes from the recipient Tg mice confirms the role of $CD8^+$ T cells in the control of transgene expression (E. Malanchère-Bres, unpublished results).

It should be noted that CpG ODN required more doses than the DNA vaccine and that the response was less complete, likely due to a weaker T-cell response, as shown by a lower frequency of IFN-γ- producing T cells. Even so, CpG ODN are powerful adjuvants for the induction of T cells regulating transgene expression in HBsAg-Tg mice.

Recently conflicting results have been reported (48) for another model of HBsAg-Tg mice (7) in which different vaccination techniques achieved neither antigen clearance nor suppression of transgene expression in the liver. This may reflect a difference in the tolerance to HBsAg in these two lineages (i.e., peripheral versus neonatal tolerance). The possibility that the control of HBV mRNA expression could be related to DNA methylation patterns is unlikely, since modifications of methylation have only been reported very early in life (41) and during tumor development (15). However, E36 HBsAg-Tg mice never developed tumors, even in advanced age. Interestingly, in the C57BL/6 genetic background, mice with high (16 μg/ml) or low (<100 ng/ml) levels of serum HBsAg have similar levels of DNA methylation, and methylation is also independent of age (49; C. Pourcel, personal communication).

In conclusion, this study has shown the efficiency of CpG ODN as an adjuvant to HBsAg vaccination to trigger specific antibodies and Th1-biased immune responses.

Vaccination of chronically HBV-infected patients has already been performed with classical recombinant vaccine adjuvanted with alum. Despite induction of efficient HBs-specific

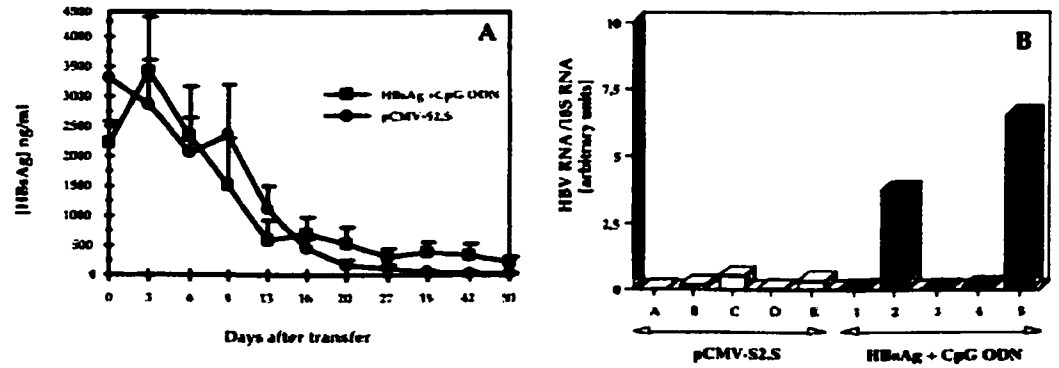


FIG. 7. Adoptive transfer of primed $CD4^+$-depleted splenocytes into HBsAg Tg-mice. $CD4^+$-depleted splenocytes, isolated from the spleens of non-Tg mice immunized with either pCMVS2.S (●) or HBsAg combined with CpG ODN (■), as in Fig. 4, were adoptively transferred into sublethally irradiated Tg mice. The Tg mice were bled, and the level of HBsAg (nanograms per milliliter) in the sera was evaluated on a weekly basis (A). HBV mRNA content (B) in the livers of HBsAg-Tg recipient mice was quantified by Northern blot analysis and PhosphorImaging 50 days after transfer of primed $CD8^+$ T cells from mice immunized with pCMV-S2.S (open bars) or with HBsAg plus CpG ODN (black bar). Each bar represents an individual value expressed as an HBV RNA/18S RNA ratio in arbitrary units.

B and T helper responses in some patients, the long-term clearance of HBV DNA was not achieved in all patients (9).

Successful immunization and protective efficacy in numerous animal models (14, 51) and induction of cellular immune responses in humans have been demonstrated with DNA vaccines (54). However, despite induction of CTL responses, problems regarding induction of antibody responses in humans remain to be resolved (28, 50). This could be particularly important for hepatitis B, for which both antibodies and cellular immune responses have been implicated in disease resolution (6). Thus, CpG ODN could represent an alternative method for modulating the immune response by combining the advantages of both classical and DNA vaccines.

ACKNOWLEDGMENTS

We thank C. Pourcel for critical reading of the manuscript and helpful discussions. We thank R. Virus (Aventis Pasteur) for generously providing purified recombinant HBsAg.

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