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Prospects and progress of DNA vaccines for treating hepatitis B

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Summary

The hepatitis B virus (HBV) is a global cause of liver disease. The preventive HBV vaccine has effectively reduced the disease burden. However, an estimated 340 million chronic HBV cases are in need of treatment. Current standard therapy for chronic HBV blocks reversed transcription. As this therapy blocks viral maturation and not viral protein expression, any immune inhibition exerted by these proteins will remain throughout therapy. This may help to explain why these drugs rarely induce off-therapy responses. Albeit some restoration of immune function occurs during therapy, this is clearly insufficient to control replication. Central questions when considering therapeutic DNA vaccination as an addition to blocking virus production are as follows: what does one hope to achieve? What do we think is wrong and how can the vaccination correct this? We here discuss different scenarios with respect to the lack of success of tested DNA vaccines, and suggest strategies for improvement.

Keywords: HBV, vaccines, therapy, DNA vaccine, electroporation

The hepatitis B virus

The hepatitis B virus (HBV) is an enveloped, partially double –stranded 3.2 kb DNA virus that binds to the cell through the recently identified receptor sodium-taurocholate cotransporting polypeptide present on the surface of hepatocytes [1-4]. After binding of HBV to the hepatocyte, the virion enters the cells through receptor-mediated endocytosis [3]. The viral lipid envelope fuses with the endosome membrane and the capsid is released in the cytosol. The capsid uses the microtubuli for transport to the nucleus where the HBV genome is released into the nucleus [3]. In the nucleus the partially dsDNA converted into a covalently closed circular DNA (cccDNA). The stability of the cccDNA in the nucleus of infected cells is central for the persistence HBV [1]. The transcription from the cccDNA generates both mRNA transcripts for the viral proteins as well as the viral RNA. The pregenome and the polymerase becomes encapsidated by the hepatitis B core antigen (HBcAg) [1, 3]. While in the capsid the viral polymerase converts the RNA pre-genome to the mature partially dsDNA through reversed transcription. The capsid presumably buds into ER/golgi to become enveloped and leaves the cell through secretory vesicles [5]. Thus, todays NUCs blocks the reversed transcription step and thereby block virus maturation [6, 7]. As a consequence, these compounds do not affect transcription of mRNAs or the pre-genome [1].

The hepatitis B virus infection

Hepatitis B is a global health concern and responsible for 1 million deaths every year [8]. Around 2 billion people have been infected by HBV, and of these 350 million people are chronically infected. The latter belong to a higher risk category for developing the liver related severe consequences such as liver cirrhosis and HCC [8].

HBV infection is most prevalent in South East Asia, China, sub-Saharan Africa and the Amazon Basin with a lifetime risk of infection of > 60%. The populations in low endemic regions such as the North America, Western Europe and Australia have < 20% lifetime risk of infection. Those living in areas of intermediate endemic regions, such as Eastern and Southern Europe, Russia, Central and South America have a lifetime risk of infection ranging from 20-60%. In developed countries the prevalence is higher among immigrants from high- or intermediate prevalence countries and those with high-risk behaviors [8].

Exposure to HBV can cause wide disease spectrum ranging from an asymptomatic infection to acute, fulminant, and chronic hepatitis. Age at infection is a predisposing factor for chronicity. The risk of developing a chronic infection is inversely related to the age at which infection occurs. Up to 90% of infants' HBV infection acquired from their mothers at birth results in chronic infection, but in adults, only 5% of acute HBV infections evolve into chronicity [9].

The chronic HBV infection can lead to liver damage (inflammation, fibrosis, and cirrhosis) and may ultimately result in liver failure or hepatocellular carcinoma (HCC). Fulminant hepatitis occurs in less than 2% of infected individuals. 15% to 40% of chronically infected individuals develop symptomatic disease in their lifetime, and up to 25% will die from directly related causes [8]. Persons with HBV infection

have a 15% to 25% more risk of premature death from hepatic decompensation and HCC.

The clinical manifestations in acute and chronic infection differ. During the acute infection the symptoms range from asymptomatic to icteric hepatitis and, in a few cases fulminant hepatitis. Most patients with chronic hepatitis B are clinically silent or have unspecific symptoms such as fatigue. Clinical symptoms develop with progression to liver cirrhosis and development of HCC. Jaundice, ascites, edema and encephalopathy may be present in decompensated cirrhosis, which further leads to worsening of the disease.

Vaccination against HBV

The first universal vaccination program for HBV infection was launched in 1984 in Taiwan [10, 11]. This vaccination regimen has been found to be at least 90% effective in preventing vertical transmission of HBV when given at birth [12, 13]. These standard HBsAg-based vaccines have also been evaluated as therapeutic vaccines in CHB patients and experimental animal models. Early data suggested some effects also as therapeutic vaccines [14, 15]. However, any antiviral effect of these HBsAg-based vaccines was only transient and failed to control viral replication [16, 17]. Hence, this type of therapeutic vaccination alone using current prophylactic vaccines is not sufficient to achieve the control over HBV. Studies in animal models have suggested that the combination of therapeutic vaccination with antiviral treatments, using viral vectors strategy and with T-cell function modulation could be explored for CHB.

Therapy for chronic HBV

Currently the approved therapeutic regimens include standard and pegylated-interferon (IFN)-α and monotherapy with available nucleos(t)ide analogues (NUCs) like lamivudine, entecavir, and tenofovir [6, 7]. IFN-alpha therapy of patients with CHB can result in HBeAg/anti-HBe seroconversion in around 30% of treated patients, but is also associated with side effects such as flu-like symptoms and severe depression. In contrast, NUCs targeting the reversed transcriptase function, are well tolerated in long-term therapies but may have a high risk of resistance development [6, 7]. The resistance rates are higher with earlier generation NUCs such as lamivudine, telbivudine, and adefovir [6, 7]. Entecavir and tenofovir are associated with low risk of resistance, but resistance development cannot be completely ruled out [6, 7]. Treatment with these compounds is efficient in inhibiting HBV replication and thereby reducing liver inflammation and, fibrosis and cirrhosis, but rarely achieve virus elimination [6, 7]. HBV reactivation upon cessation of therapy is the major issue with these treatments. As these drugs only block maturation it is unusual to achieve a sustained control of HBV replication off-therapy.

Alternative adjuvant therapies

Novel immunotherapies may target HBV-infected hepatocytes by many different modes of action. For example, a recent study proposed that activation of lymphotoxin-β receptor (LTβR) of HBV-infected cells can be used as a therapeutic alternative capable of mediating degradation of cccDNA in infected hepatocytes without hepatotoxicity [18]. In addition, immunotherapeutic approaches may boost HBV-specific T-cell responses, or stimulating the liver specific innate immune response (toll-like receptor agonists and cytokine delivery). Immunomodulation by exploiting the robust antiviral efficiency of cytokines like TNF-α, IFN-α, IFN-γ,

Interleukin-1β thereby increasing the HBV specific innate immune response have also been explored [19-21]. An interesting example is the blocking of the programmed death receptor-1 (PD-1), which has been explored in chimpanzees with chronic HCV infection [22] We will herein focus on the potential role of therapeutic DNA vaccination as an alternative adjuvant therapy to be added to the antiviral NUC backbone.

Therapeutic DNA Vaccine for HBV

Is there any rationale for therapeutic DNA vaccination alone?

In the chronic viral infection, a first so called tolerant, or non-inflammatory, phase is characterized by a higher viral load, normal liver enzymes, and lack of inflammation by histology ([23, 24]; Figure 1). There is a debate whether this can be designated as a tolerant phase, suggesting that either there are none, or non-functional, HBV-specific T cells in the liver. Studies starting in the 1990s showed that the hepatitis B e antigen (HBeAg) could pass the placenta and induce tolerance to HBeAg and hepatitis B core antigen (HBcAg), in non-HBeAg transgenic littermates borne to HBeAg-transgenic mothers [25]. This may mimic the situation in vertical transmission in humans, where >90% of infants born to HBeAg positive mothers become chronically infected [8]. Thus, a gap in the HBV-specific T cell repertoire should thereby be present at birth, paving the way for the chronic infection [25]. New data suggest that the HBV infection in fact may support the maturation of the host immune system [26]. Additional data also suggest that functional HBV-specific T cells may in fact be present that actively fight the infection in the absence of ALT elevations [27]. It has been suggested that the anti-viral activity does not result in a detectable elevation of ALT levels rather, it may be the unspecific inflammation that

causes the ALT elevations [24, 27]. Alternatively, the absence of ALTs could be due to immune escape or non-inflammatory responses (Figure 1).

In this early period of the HBV infection therapeutic DNA vaccination may have a place, unless the environment is strongly inhibitory for T cells. At later stages of the disease, during the inflammatory phase is when therapies are most effective (Figure 1). During this period IFN-based therapies are able to induce HBeAg/anti-HBe seroconversion at rates of around 30% of patients, although the addition of NUCs do not seem to improve response rates [6, 28]. Therapy is well indicated in this phase, as patients with elevated liver enzymes and viral replication are those most likely to develop a progressive disease [8]. The preferred therapies today are second generation NUCs such as entecavir and tenofovir, which both can be used for long periods (years) without development of resistance [6]. NUC therapy results in a significant drop in the viral replication concomitant with normalization of ALT levels. Thus, as the viral replication is controlled the inflammatory signals are reduced, despite the presence of many viral antigens. It has been shown that NUC therapy results in a detectable restoration of HBV-specific T cell responses at various time points during therapy [29, 30]. Hence, viral replication itself seems to promote inflammation and impair HBV-specific T cell responses. However, at cessation of therapy the viral replication and inflammation rebounds in almost all patients suggesting that the therapy-induced restoration of HBV-specific T cells is not sufficient to control viral replication [6]. This leads us into the concept of therapeutic vaccination. What would be the result if the restored but insufficient HBV-specific T cell-responses, could be successfully boosted and expanded by repetitive DNA vaccinations? The aim would be to expand, or induce de novo, T cells that help

control the viral replication after NUC therapy stop. Hence, giving the host back the control of the infection.

The rationale for development of therapeutic vaccines for chronic HBV infection

A substantial number of attempts have been made to develop functional therapeutic vaccines for chronic HBV infection, including various types of viral vectors such as retroviral and adenoviral, as well as plasmid DNA [31-37]. The rationale for a therapeutic vaccine is to activate, or boost, the patient's own immune system to take control of the infection and ultimately control it. It is well documented that individuals who resolves an acute infection mount a vigorous and multi-specific T helper (Th) and cytotoxic T cell (CTL) response to HBcAg (nucleocapsid), polymerase and the HBsAg (surface/envelope) proteins, whereas individuals that progress to a chronic infection only have weak or undetectable Th- and CTL responses to HBV antigens [38-40]. The most striking evidence that a therapeutic vaccine may work for chronic HBV infection is the fact that immunocompetent adult individuals infected with HBV may spontaneously seroconvert from HBeAg-positive to anti-HBe and HBsAg-positive to anti-HBs [8]. This is intimately associated with immune control of the chronic HBV infection unless the individual is immunosuppressed. Hence, a functional HBV-specific immune response is believed to be responsible for control of HBV infection. So what are we aiming for with a therapeutic vaccine and why do we need such vaccine? We aim at activating or reactivating the patient's own dysfunctional or weak cellular immune responses to HBV. This can be achieved by using different types of vaccines in combination with adjuvants and delivery devices. In addition, it has been well documented that the current antiviral treatment composed of pegylated IFNα and/or a NUC will not clear

or cure the HBV infection [6-8]. Notably it will significantly reduce the disease burden caused by the infection [41, 42]. Thus, it is obvious that an important component is missing in todays HBV therapy. The current antiviral drugs must be given continuously to the infected patients since disruption of treatment is associated with recurrence of viral replication [7]. The reason for this is the persistence of episomal HBV cccDNA in the nucleus of infected hepatocytes, which is believed to be responsible for HBV reactivation [1, 3]. Another complicating factor is that the HBV genome, or parts thereof, can be integrated into the host genome during long-lasting viral replication that may promote carcinogenesis [1, 3]. Thus, the goal of therapeutic vaccination is restoration of an antiviral HBV-specific T cell response that favor control and/or clearance of hepatocytes containing HBV cccDNA, and maybe even cells containing integrated HBV genome sequences. Next we will discuss alternative designs for therapeutic HBV vaccines.

Which HBV antigens should be used in therapeutic vaccination?

There are seven proteins expressed from the HBV genome. Three forms of the hepatitis B surface antigen (HBsAg) are produced, the large (L) HBsAg containing preS1, preS2, and S, the middle (M) HBsAg containing preS2 and S, and the small (S) HBsAg containing only S. All these form lipid-containing virus like particles (VLPs). The SHBsAg is currently dominating vaccine component in today's prophylactic HBV vaccines. As the infected hepatocytes overexpress HBsAg in chronic HBV infection it is doubtful whether HBsAg is a viable therapeutic vaccine candidate. This is based on the fact that soluble antigens are highly efficient in suppressing T cell responses. In addition, the high levels of HBsAg will effectively neutralize the antibodies to HBsAg (anti-HBs) that is produced and that may prevent spread of the

infection. However, one may consider including vaccines containing preS1 and/or preS2 to improve the levels of neutralizing antibodies as one vaccine component [43].

Two proteins are expressed from the preC/core open reading frame (ORF), the hepatitis B e antigen (HBeAg) and the hepatitis B core antigen (HBcAg) [3]. HBeAg is a secretory protein expressed from the preC start codon resulting in the secretion of a post translationally modified protein of around 150 aas. HBeAg is most likely secreted as a monomer although the structure of serum HBeAg has not been determined [3]. In contrast, HBcAg is an 180 aa protein that assembles into the capsid around the viral RNA pre-genome. HBcAg particles have been found to be highly immunogenic both as particle-based vaccines and as DNA vaccines [33, 34, 44]. Thus, HBcAg is certainly an excellent candidate to be included in a therapeutic vaccine. The possibility to use HBeAg in genetic vaccines has not been fully explored. It should be noted that both HBcAg and HBeAg are genetically stable proteins.

The X-protein is a non-structural protein expressed in infected cells and has been implicated in cell transformation and cancer development. That simple fact that the X-protein may have transactivating properties has most likely discouraged its use in vaccines [3]. It may be speculated that the X-protein may be the cause of the rare cases with HCC development occurring only a few years after infection. Thus, the X-protein is also a protein that not has been fully explored as a therapeutic vaccine component.

Finally, the comparatively large polymerase (pol) protein with reversed transcriptase (RT) activity is a structural protein also present in the infected cell [1, 3]. The pol protein converts the partially dsDNA genome into the cccDNA that is stably maintained in the nucleus. The RT function of the pol protein also converts the

encapsidated RNA pre-genome into the partially dsDNA that completes the maturation of the HBV virion [1, 3]. Overall, pol is certainly an interesting component in a therapeutic vaccine against HBV. However, we also know that a certain degree of genetic variability in the pol is accepted with retained function, as evidenced by the appearance of drug-resistant virus.

The role of inflammation in confusing functional T cells

Quite surprisingly, it has been shown that in chronic HBV infection, there may in fact be a similar amount of HBV-specific T cells in patients who control, and those who do not, control viral replication [27]. Hence, this raises the possibility that the specific T cells are in fact entering the liver but the local milieu prevents them from having antiviral activity (Figure 1). If this is the case, can it be envisioned that simply increasing the number of specific T cells by a highly effective vaccination will overcome the hostile environment and restore function (Figure 2)? This should to be tested and explored in *in vivo* model systems.

As an alternative, can a simple modulation of the milieu restore T cell function? Here a number of check-point inhibitors such as anti-PDL1 and CTLA4 have been tested [22, 45]. In various model systems they can restore function in human T cells from HBV infected individuals, and may help restore T cell function in cancer therapies. As an example, it was recently shown that blockade of PD-1 signaling enhanced the restoration of T cell responses in chronic woodchuck hepatitis B virus (WHV) infection treated with entecavir and DNA vaccination [46]. This is the first example that a triple combination therapy seems attractive in an infectious model using a hepadna virus.

Clinical Efficiency

Overview of preclinical studies

Several preclinical studies (mice and chimpanzees) in mid 1990´s could conclude that plasmid DNA could be used to deliver the HBsAg to induce specific T cells [47-49]. The HBsAg synthesized *in vivo* by plasmid transfection induced anti-HBs that recognize different epitopes of both S and preS2 components of the HBV envelope protein. Importantly, the anti-HBs levels reached a protective level known to prevent HBV infection and hepatitis. In chimpanzees the response was transient and dose dependent with high doses of DNA (2mg) [47], whereas single DNA vaccination in mice led to anti-HBs lasting up to 6 months [48]. This strategy was further evaluated in animal models including the woodchuck HBV virus (WHV) model. This demonstrated that the immunogenicity of a naked DNA vaccine could be augmented by co-delivery of cytokine adjuvants such as IL-2, and the bacterial HSP70 gene [50]. Fazio and coworkers showed that a single intramuscular in-utero anti-HBV DNA immunization at two-thirds of pig gestation produces, at birth, antibody titers considered protective in humans [51]. Upon a subsequent boost a long-term immune memory could be seen in a 2 years follow-up.

As plasmid DNA is comparatively simple to produce and stable over time, it has become a popular vaccine tool. The major limitation is, of course, delivery and uptake of the plasmid DNA in larger animals including humans. However, due to the ease of producing plasmids it became apparent that one could use several antigens and even mix several plasmids, such as HBcAg, HBeAg, and HBsAg to broaden the T cell responses [52-54].

Many reasons suggest that HBcAg can be a key component of a DNA-based vaccine for chronic HBV. But how do you maximize immunogenicity of HBcAg? We have

analyzed the immunogenicity of HBcAg in various forms, recombinant HBcAg and HBeAg [44], a retroviral vector expressing a fusion variant of the HBcAg protein [55, 56], and plasmid DNA [31, 34]. We have found that HBcAg can be sensitive to the delivery technique when used as genetic immunogen, showing the best immunogenicity when delivered intra-muscularly (im) [34]. A simple way to improve immunogenicity was to codon optimize the plasmid and deliver using electrotransfer or electroporation (ET/EP). ET/EP transiently destabilize the cell membrane allowing for improved uptake of the plasmid [57]. In addition, *in vivo* ET/EP induces a transient and reversible local inflammation, which further promotes immunogenictity [58]. Delivery can be even further improved by combining *in vivo* ET/EP with a targeted high-pressure injection [31]. When co-administering a codon optimized HBcAg plasmid, with a plasmid expressing IL-12, using these combined delivery techniques, this primed an *in vivo* functional T cell response in HBeAg-transgenic (Tg) mice [31]. Importantly, the HBeAg-Tg model has a dysfunctional T cell response to HBcAg and HBeAg that better resembles the dysfunctional immunological state of chronically infected HBV patients, with a limited HBV-specific T cell repertoire, or simply lack function or ability to recognize a target.

It was recently shown in the woodchuck hepatitis (WHV) model that a DNA prime-adenovirus boost immunization using WHV surface antigen (WHsAg) and WHcAg combined with direct antiviral treatment was promising [46, 59]. The animals remained WHV-negative after interruption of the antiviral treatment and developed anti-WHV antibodies [59]. A triple-combination therapy with DNA vaccination encoding the WHcAg and WHsAg, programmed death-ligand 1 blockage and antiviral treatment could further result in sustained immunological control of viral

infection, specific antibody response, and complete viral clearance (with undetectable levels of cccDNA in the liver) in one out of three animals tested [46].

Clinical testing of therapeutic DNA vaccines for chronic HBV infection

The hypothesis whether DNA vaccination can specifically activate immune responses in chronic HBV carriers who do not respond to antiviral therapies, e.g. therapeutic vaccination, was addressed by clinical trials in chronic HBV carriers [50, 60, 61]. They examined the DNA vaccine comprised of pCMV-S2.S DNA encoding the small (S) and middle (preS2 + S) proteins of the HBV envelope (ayw subtype) previously evaluated in mice and chimpanzee models (Table). The trial included 10 patients with chronic active hepatitis B non-responder to approved treatments for HBV infection, they were given 4 intramuscular injections of 1 mg of the DNA vaccine. A hallmark of the immune response in chronically infected HBV patients is absence, non-functional, or exhausted T cells to HBV. However, after 3 DNA injections, the authors noted a transient restoration of T-cell responsiveness, along with NK cell activation and anti-HBV antibody responses (Table). One patient (HB21) was particularly interesting, who controlled HBV DNA and seroconverted in the HBeAg/anti-HBe system during follow up [60]. The following events occurred in chronological order, vaccinations at weeks 0, 2, and 4, HBV DNA flare at week 4, an ALT flare at week 5, HBcAg-specific T cell proliferation at weeks 5 (weak) and 9 (strong), HBsAg-specific T cell proliferation at week 11. Thus, first a peak in HBV replication followed by an ALT flare and weak HBcAg-specific T cell activation, and then a transient strong T cell response at weeks 10 and 11 [60]. It is tempting to speculate that the DNA vaccination was indeed responsible for the T cell activation and the subsequent HBeAg seroconversion. In fact, this mimics what we noted in a

chimpanzee vaccinated with a retroviral vector expressing a modified HBcAg, and who displayed ALT elevations, controlled HBV replication and who seroconverted in the HBeAg system after vaccination [35]. Thus, although both these primates did control the HBV replication, it cannot be excluded that this would have happened without the DNA/RNA vaccinations.

Since T cell dysfunction is most likely more pronounced during a high viral load, it is desirable to reduce the viral load before administering the vaccine. This may release a bit of the inhibiting effects that the replication itself has on the immune response, as suggested by some studies describing some T cell restoration during NUC therapy. One study in 39 patients on lamivudine with or withour DNA vaccination suggested a beneficial effect of the vaccine [61]. A prolonged NUC therapy prior to vaccination should improve the chances of inducing, or reactivating, an HBV-specific immune response and eventually control the infection, and maybe even eliminate all/most hepatocytes with cccDNA. In a recent randomized phase 1/II trials that included 70 patients who received long-term NUC treatment of chronic HBV replication prior to therapeutic vaccination with a HBsAg DNA-based vaccine, unfortunately failed to show any efficiency [62]. The vaccine had no additive effect on viral reactivation after the NUC discontinuation [62]. Moreover, the study of ex vivo peripheral T cell responses did not show significant vaccine-specific IFN-γ responses during the trial. There are many reasons why this trial may have failed. The two key reasons are most likely the use of HBsAg as the vaccine antigen, and not enhancing the uptake of the plasmid DNA by any means. HBsAg is produced in high quantity in chronically infected HBV patients. Thus, these T cells are most likely the hardest to activate/reactivate. In addition, the injected DNA was most likely only poorly taken up by cells resulting in a low antigen expression and a poor

immunogenicity. In conclusion, this study highlights the difficulty to evaluate findings from early uncontrolled phase I clinical trials, and the importance to continue into controlled clinical trials to understand the effects.

Expert Commentary

How should future DNA vaccines for chronic HBV be designed and tested?

Based on everything we know today, it will be difficult to cure chronic HBV infection. So what rationale can we used to improve our chances for a success? There are most likely three things that are instrumental to success: the vaccine antigen, the delivery technique, and which patients that we treat.

1. The vaccine antigen

So how should the vaccine antigen be selected? First, it should be an antigen that does not has a limited intra- and interhost sequence variability, it should be highly immunogenic as a DNA vaccine, and, most importantly, it should be an antigen whose expression levels is reduced by NUC therapy. For example, HBsAg levels in serum is only marginally affected after years of NUC therapy, suggesting that any inhibitory effect that the antigen will have on the immune response will be maintained even during years of therapy [7]. Also, HBsAg has been shown to undergo mutations to escape the host immune response [63]. However, as neutralizing antibodies are directed to HBsAg, this may well be an interesting component to add to a therapeutic vaccine regimen. This may further reduce infection of new hepatocytes, when HBsAg levels are reduced. In contrast, serum levels of HBcAg have been shown to correlate with the decrease in serum HBV DNA levels [64, 65]. Also, serum HBcAg levels

have been shown to correlate with intra-hepatic cccDNA levels [66]. More importantly, the decrease in serum HBV DNA was also strongly associated with a reduction in both cytoplasmic and nuclear expression of HBcAg in hepatocytes [65]. Thus, HBcAg is one of the antigens that do decrease in expression levels during NUC therapy. This is not difficult to explain since the blocking of RT function by NUCs reduces the number of newly infected cells, and hence, the number of cells expressing HBcAg will not increase, but rather decrease. In contrast, the overexpression of HBsAg will remain from the already infected cells seemingly unaffected by the therapy, thus maintaining the HBsAg levels in serum. Subsequently, it is likely that HBcAg-specific T cells are more easily activated/reactivated by a DNA-based vaccine strategy. This is most likely true also for the pol protein.

2. The delivery technique

There is absolutely no idea to perform clinical trials without any tool that improves either, or both, the cellular plasmid uptake and a local inflammation. There are several tools that can be applied. Different forms of nanoparticles or liposomes may well improve DNA uptake in humans, as for example the poloxamer-based DNA vaccine that showed efficiency in controlling recurrence of cytomegalovirus in transplant patients [67]. The use of needle-free injector seems to be more convenient, but only slightly more effective than a regular i.m. injection with respect to DNA delivery in humans [68, 69]. Another technology is *in vivo* ET/EP that has been applied to DNA vaccines against chronic hepatitis C virus infection and human papilloma virus infection [70, 71]. In brief, in vivo EP/ET adds an electrical current over the injection site whereby the cellular membranes become destabilized and thereby permeable and the DNA uptake increases. In addition, the EP/ET induces a local inflammation that

most likely helps to recruite immune cells essential for activation on an antiviral response [57, 58]. This shows that effective immune responses can be induced in humans with DNA, given that the delivery issue has been properly addressed.

3. The clinical trial design

The clinical trial design is pivotal for success given the rather tough conditions in the chronically infected host that the vaccine-induced immune responses are expected to function. The patients should not be those that have failed all other therapies, these are highly likely to fail also in vaccine trials. The type of patient that should be targeted in the early trials are those that are likely to respond to IFN therapy, as in IFN therapy a state of host control of the infection is achieved during a successful therapy [39]. Thus, patients who at start of NUC therapy had elevated ALTs and who responded well to the NUC therapy with stable low viral loads, and HBsAg levels <1000 IU/mL. The patients should have been on stable NUC therapy for at least one year to allow for some recovery of the HBcAg-specific immune responses [29, 30] (Figures 1 and 2). As previously stated, hepatic expression of HBcAg decreases with the NUC therapy, whereas the HBsAg expression levels do not. Hence the reasons to choose those with a lower HBsAg load. How many vaccinations should be given? This is hard to predict, and may well vary from patient to patient. The best criteria for a successful therapeutic vaccine regimen, with respect to immunogenicity, could be that a *de novo* activation of HBV-specific T cells is induced after "x" number of vaccinations, determined as for example, as a specific proliferation, a certain number of IFNγ-producing T cells per million PBMC, or a certain frequency of tetramer/pentamer positive cells. If these pre-set criteria have been reached for the individual patient, then the NUC therapy may be stopped (Figure 2). If the induced

immune responses are antiviral and functional then the HBV replication should set stably at a lower level than before start of the NUC therapy. This is most likely how we would design our next clinical trial of a therapeutic DNA vaccine for chronic HBV infection.

Five-year view

The coming five years will most likely be truly exciting for HBV therapy. We can expect a number of experimental clinical trials including new NUCs, other small molecules targeting various steps in the HBV life-cycle and in particular the cccDNA, and most importantly, various immune modulating regimens, including therapeutic vaccines. Some of these trials will for sure include DNA-based vaccines and will in many cases, hopefully, be designed to include the easy to treat patients. Thus, we will in the next five years most likely see the same "warp-speed" development in experimental clinical trials as we just have experienced for chronic HCV. This is good for the patients, so that they may live long and prosper.

Key issues

- Current therapies for chronic HBV are life-long as they do not allow for a complete restoration of the host immune response that can control HBV.
- The future for chronic HBV will be the same as for HCV, which is combination therapies, however most likely also needing an immune modulatory component.
- Future combination therapies should result in off therapy responses with a sustained control of viral replication.

- DNA vaccines must be delivered in the best possible way to ensure plasmid uptake and immunogenicity.
- Care should be taken to monitor the patients during the vaccination therapy to reduce the risk for severe immune-associated adverse events such as fulminant hepatitis.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Reference annotations

** Of interest*
*** Of considerable interest*

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Figure 1. Cartoon showing the possible immune events occurring in the HBV infected liver before (upper half) and during antiviral therapy with NUCs. In brief, Tregs may play dual roles in disease progression. Tregs may inhibit specific T cells and NK cells, as well as promote fibrosis and inflammation through activation of Th17 cells that in turn activate hepatic stellate cells (HSCs). The HSCs activate Kupfer cells and macrophages adding to the local inflammation. This result in an environment that prevents a proper function of virus-specific T cells and that promotes liver disease (upper half). In contrast, during NUC therapy the viral replication is decrease through the inhibition if release of infectious virs and reduction of newly infected cells. This has a profound effect on the inflammation and development of fibrosis. Thus, the levels of HBc-related antigens (HBcrAg) decrease and the T cells become functional again (slowly). The cytokine environment becomes more antiviral. Now an activation of HBcrAg-specific T cells may be effective through therapeutic DNA vaccination (lower half).

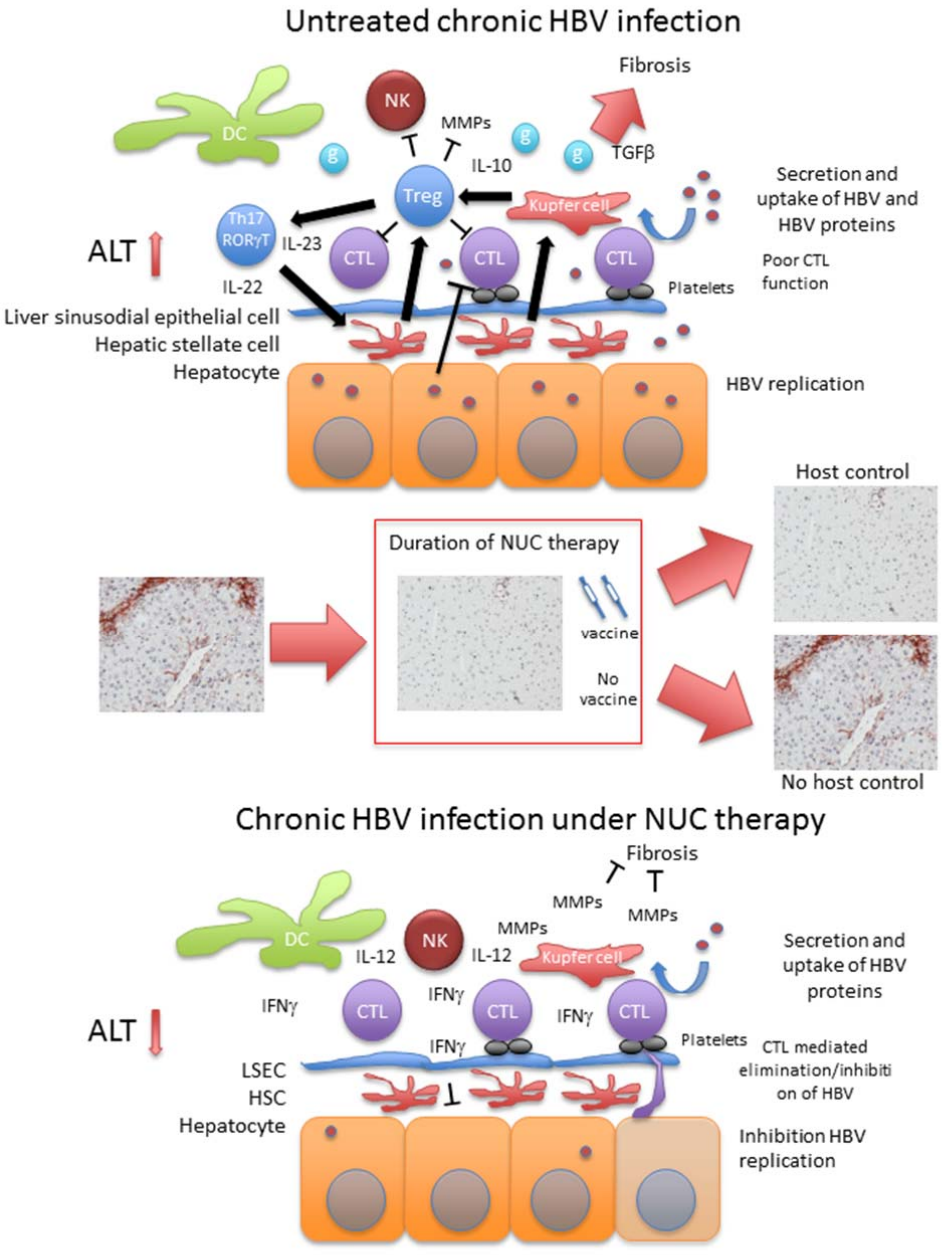

Figure 1

Figure 2. Serum markers and inflammation in chronic HBV during chronic HBV infection (a) and the proposed timing of therapeutic vaccination in relation to the concept of inflammatory state preventing the function of T cells (b) or the concept of a T cell tolerance (c).

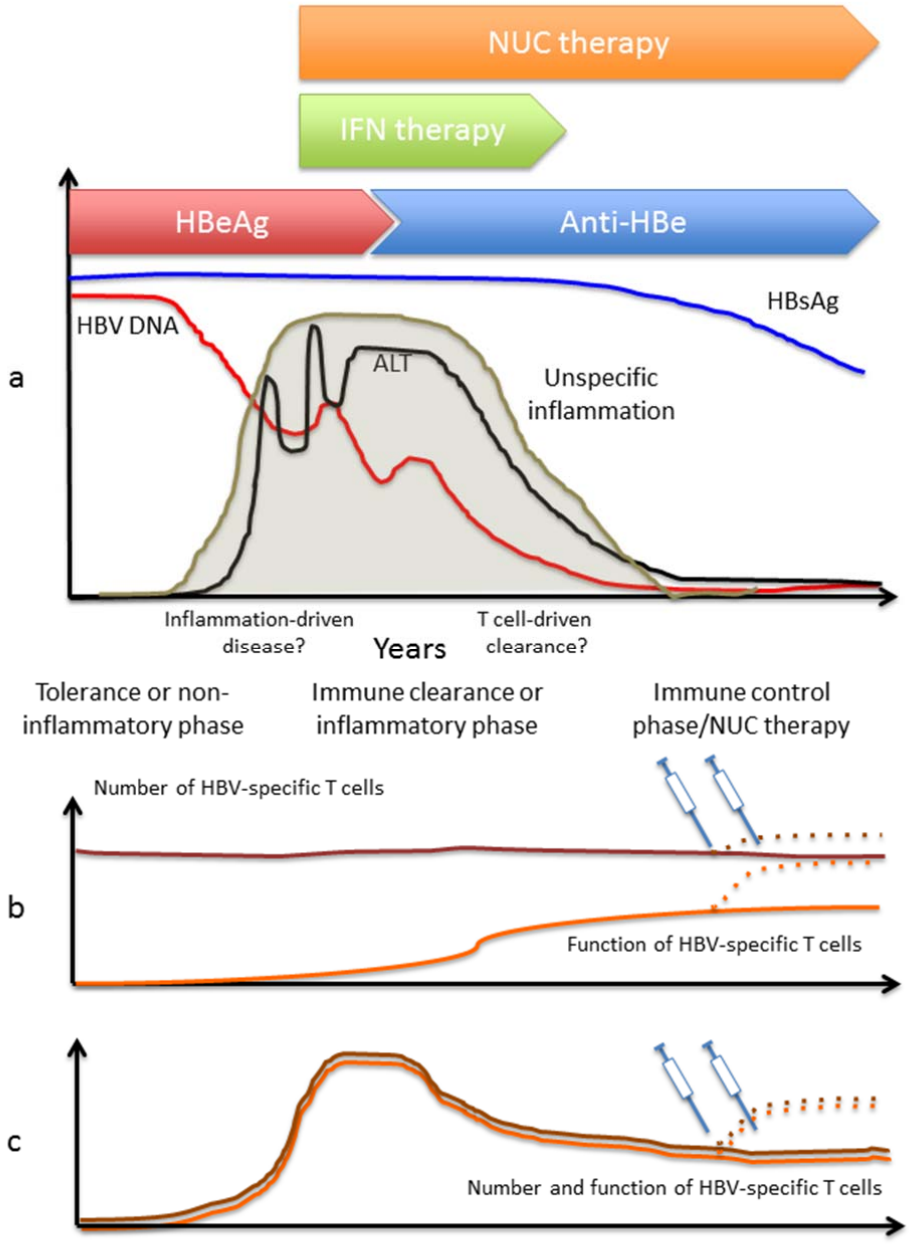

Figure 2

Table. Over view of clinical trials using naked DNA for HBV.

Patients	Trial design	DNA vaccine antigen	Safety/Effect	Outcome
Chronic HBV (n=10) [60]	Phase I safety/i.m. injection	HBsAg (pres2+S)	Safety good/reduction in HBV DNA	Transients immunological and virological effects
Chronic HBV on NUC (n=70) [61]	Controlled trial/i.m injection	HBsAg	Safety good/no effect	No restoration of HBsAg-specific T cells
Chronic HBV n=39 [71]	DNA alone, LAM alone, and LAM DNA	HBsAg and IL-2/IFNγ-fusion	DNA/LAM group showed time points with improved control of virus and immune responses	A statistical effect only at single time points