공학박사학위논문

Preclinical study on a novel DNA vaccine (HB-110) for the treatment of chronic Hepatitis B virus infection

B형 간염 치료용 DNA 백신(HB-110)의 전임상 연구

2014년 8월

서울대학교 대학원
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Preclinical study on a novel DNA vaccine (HB-110) for treatment of chronic Hepatitis B virus infection

A Thesis

Submitted to the Faculty of Seoul National University

By

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ABSTRACT

Interferon-α immunotherapy and chemotherapeutic agents including Lamivudine, Adefovir and Entecarvir have been developed so far for the treatment of Hepatitis B Virus (HBV) infection and are widely used. Interferon-α is associated with serious side effects, while chemotherapeutic agents, although being generally safe and effective at inhibiting virus proliferation, also show toxicities after long-term use. Importantly, chronic administration of chemotherapeutic agents is often associated with occurrence of resistant virus in patients. Also, recurrence of HBV is common once these agents are discontinued. With the purpose of providing an HBV therapy with efficacy and safety superior to existing therapies, in the present study a therapeutic DNA vaccine was developed for the treatment of patients with HBV infection. Safety and efficacy of the lead compound, HB-100, was demonstrated in a pilot clinical study conducted in Ukraine and Lithuania. Later, an optimized compound, HB-110, with higher efficacy and economic productivity compared to HB-100 was developed.

A production process for HB-110 was developed, and preclinical studies were conducted including physicochemical/biological characterization, stability, toxicity and pharmacokinetics (PK). HB-110 is a DNA vaccine comprised of 3 different naked plasmid vectors, which express HBV antigens and a mutant IL-12. *Escherichia. coli* DH5α cells were transfected with the plasmids to produce production cell lines for each plasmid. Production cell banks of each cell line were established and characterized for genotype, phenotype and adventitious contamination. Production processes using the cell lines were established for each plasmid, and plasmid material with purity higher than 90% supercoiled monomer could be obtained by a combination of a glycerol-based feeding strategy during the culture process and incorporation of TAA chromatography in the

purification process. In accordance with regulatory guidelines for gene therapy products, physicochemical and biological characterizations were performed for HB-110 produced using the established production process, and quality test methods were established for the quality control and release testing of clinical-grade material. Toxicity evaluation of HB-110, conducted in rats and monkeys at KIT (Korea) and Ina (Japan), demonstrated that HB-110 was safe, with a NOAEL $\geq$ 4 mg/kg. A PK study conducted in a mouse model showed a half-life of 1.9 minutes and AUC of 103 ug min/ml after IV administration. Residual HB-110 was detected in the muscle at the site of administration until approximately 11 days after IM administration, whereas in other organs HB-110 levels fell below the limit of detection (0.01 pg/mg tissue) after 8 hours post-administration. Based on the data obtained from the preclinical studies, an investigational new drug application (IND) was submitted to and approved by the KFDA, and a phase 1 clinical study involving subjects with HBV infection was conducted and completed at The Catholic University of Korea Seoul St. Mary's Hospital.

With the purpose of further enhancing the efficacy of HB-110, electroporation studies in mice were conducted which showed a sharp increase of S antigen gene expression and enhancement of cellular immune response by a factor of about 1.7 to 3 times depending on the antigen. The electroporation method is planned to be incorporated in future phase 2 clinical study protocols to further enhance the clinical efficacy of HB-110.

Keywords: HBV (Hepatitis B virus), Therapeutic DNA vaccine, Cell-mediated immunity, Electroporation

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LIST OF ABBREVIATIONS

ADV	Adefovir dipivoxil
ALT	Alanine aminotransferase
ccc DNA	Covalently closed circular DNA
CHB	Chronic hepatitis B
CMC	Chemistry, manufacturing and control
CTL	Cytotoxic T lymphocytes
DI	Direct injection
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Interferon-r-enzyme-linked immunosorbent spot
EP	Electroporation
FDA	Food and Drug Administration
GMP	Good manufacturing practice
HBc Ag	Hepatitis B core antigen
HBe Ag	Hepatitis B e antigen
HBs Ag	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
IL-12	Interleukine-12
IRES	Internal ribosome entry site
KIT	Korea institute of toxicology
LAM	Lamivudine
MCB	Master cell bank
MHC	Major histocompatibility complex
NVR	Nonvirological responders
OC	Open circular form
OVA	Ovalbumin
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
RAPD	Random amplified polymorphic DNA
RT	Reverse transcriptase
SAE	Serious adverse events
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TA	Tibials anterior
TAA	Thiophilic/aromatic adsorption
TFF	Tangential flow fitration
Th1	Type 1 T helper cell
VR	Virological responders
WCB	Working cell bank
WHO	World health organization

I. INTRODUCTION

1.1 Hepatitis B virus

1.1.1 Structure of HBV

Hepatitis B virus (HBV) is a member of the hepadnaviridae family, which includes duck, ground squirrel and woodchuck hepatitis viruses. The structure of these viruses is very complex, approximately 3.2 kb in size and mostly in double strand. Four overlapping open reading frames are encoded in the genome [1]. HBV virions are double-shelled particles, 40 to 42 nm in diameter, with an outer lipoprotein envelope that contains three related envelope glycoproteins [2]. Important virus proteins include surface antigen (HBsAg), core antigen (HBcAg), e antigen (HBeAg) and reverse transcriptase (RT). HBsAg has three different types, which are small HBsAg, middle HBsAg and large HBsAg that are involved in inducing immune response and attachment to the host cell [3]. They are serologic markers first detected in HBV patients, and HBV infection can be diagnosed when they are detected for more than 6 months. HBcAg is comprised of 185 amino acids and is involved with nucleocapsid assembly. This protein is not detected in the blood of infected person and purified in a HBV infected liver cell. Hepatitis B e antigen has it's name from "early" appearance during an acute infection. This antigen is detected in infected persons by blood test and generally associated with high virus replication. RT is a virus polymerase that converts RNA to DNA in the course of virus replication and is similar to the reverse transcriptase observed in retroviridae like HIV [4].

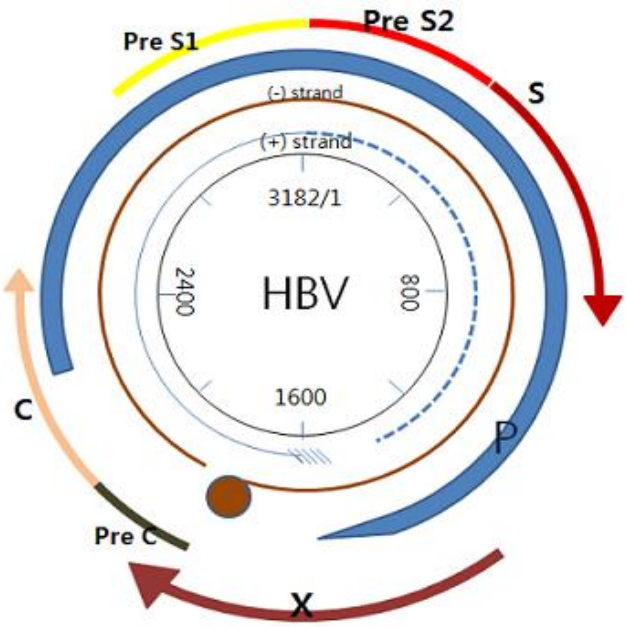


Fig.1. HBV DNA genome [5]. Schematic representation of the circular, partially double-stranded HBV DNA genome. The pre-s1/s2/s ORF encodes the three viral surface proteins, the pre-c/c ORF encodes the e antigen and the core antigen, the P ORF encodes the viral polymerase and reverse transcriptase, the X gene encodes a small protein that is essential for viral replication.

1.1.2 Life Cycle

HBV infection can be observed in some nonhuman primates but the most well-known host is human [6]. This virus is comprised of a very complicated life cycle, which includes attachment and infection, replication, assembly and budding.

During the attachment and infection, HBV mostly infects human hepatocytes and attaches to the cell surface receptor, possibly a member of ovalbumin family. When the virus membrane is fused with the host cell membrane, the virus core is released to the cytoplasm, where the partially single-stranded HBV DNA is converted to a fully double-stranded DNA by viral reverse transcriptase. During the replication, the double-stranded HBV DNA enters the nucleus and is ligated by cellular ligases to form circular episome. Since HBV has no integrase activity, HBV episome is not generally inserted in cell genome. This viral DNA is transcribed by cell RNA polymerase II. A full-length cDNA has complete genetic information of this virus. After the transcription, viral protein is synthesized in the cytoplasm. During the virus assembly and budding, full-length cRNA is encapsidated in the cytoplasm by the core protein and transcribed to partially double-stranded DNA by virus reverse transcriptase. Then the virus acquires the surface proteins by budding through membranes and ready to infect new host cells [7,8].

1.1.3 Transmission

HBV is transmitted by body fluids, such as blood, saliva and semen, similar to HIV, but 50-100 times more transmittable than HIV. The virus is most highly concentrated in blood and serous fluid and less so in saliva, semen and other fluids. Transmission by tear, sweat or urine has not been reported yet [9, 10].

The route of HBV transmission is very different depending on the region. In developing countries, HBV is transmitted by perinatal mother-to-child transmission or between infants

living in the same household. Perinatal transmission is very common, occurring in 70-90% when mother is HBsAg and HBeAg positive and child is not vaccinated. Since perinatally infected infants do not have enough immunological competence to remove the virus in the early stage, more than 90% of them develop chronic HBV infection. In developed countries, however, mother-to-child infection is very rare due to the HBV vaccination program for newborns. Instead, unprotected sexual contact, blood transfusion and shared use of injective equipment are the primary routes of transmission. Healthcare workers who are exposed to the blood and other body fluids of infected patients are at high risk of infection [11].

1.1.4 Epidemiology

HBV was first discovered in 1966 and confirmed as the cause of infection by Dane et al. who isolated complete hepatitis B virion from hepatitis B patients in 1970 [2]. Hepatitis B is detected around the world, but the prevalence varies widely depending on the region. It appears in adolescence or adulthood in developed countries, compared to Asia, sub-Saharan Africa and South Pacific, where the prevalence is very high and mother-to-child transmission or human -to-human contact in the childhood is the most common route of infection.

According to the World Health Organization (WHO), 2 billion people are infected with HBV, and 5% of world population (350 million) is suffering from chronic hepatitis B (CHB) [12]. They are at high risk of developing to liver cirrhosis or liver cancer, and 500,000-700,000 people die of HBV infection every year [13]. From the survey conducted by the Korean National Institute of Health involving the people chronically infected with HBV, the Korean prevalence was 5.1% among men, 4.1% among women and approximately 4.4% in general [14].

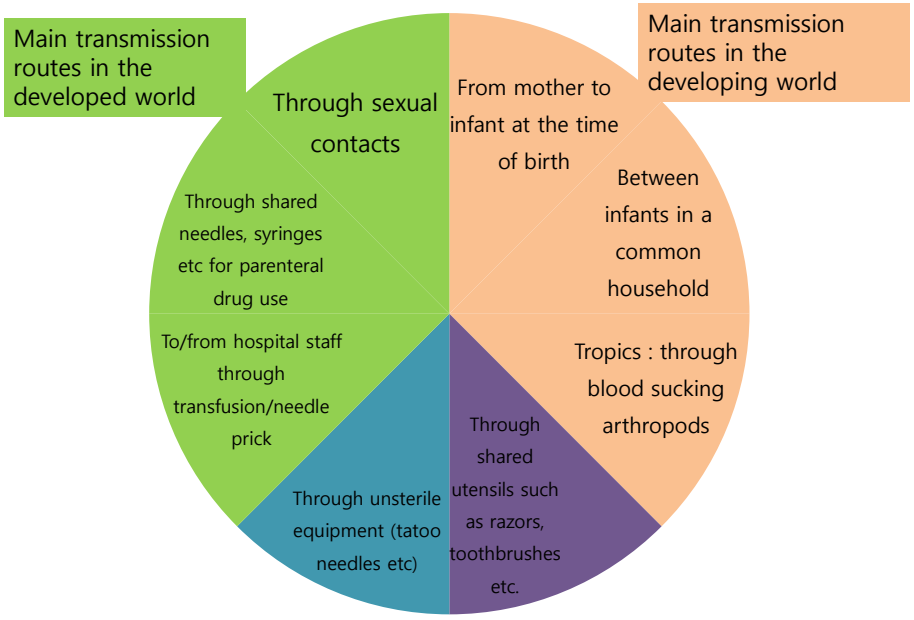


Fig.2. HBV- routes of transmission. Main route of HBV transmission was compared between developing and developed countries. Mother-to-infant infection was the main route of transmission in developing countries, while sexual contact, blood transfusion and shared use of needles were the main route of transmission in developed countries.

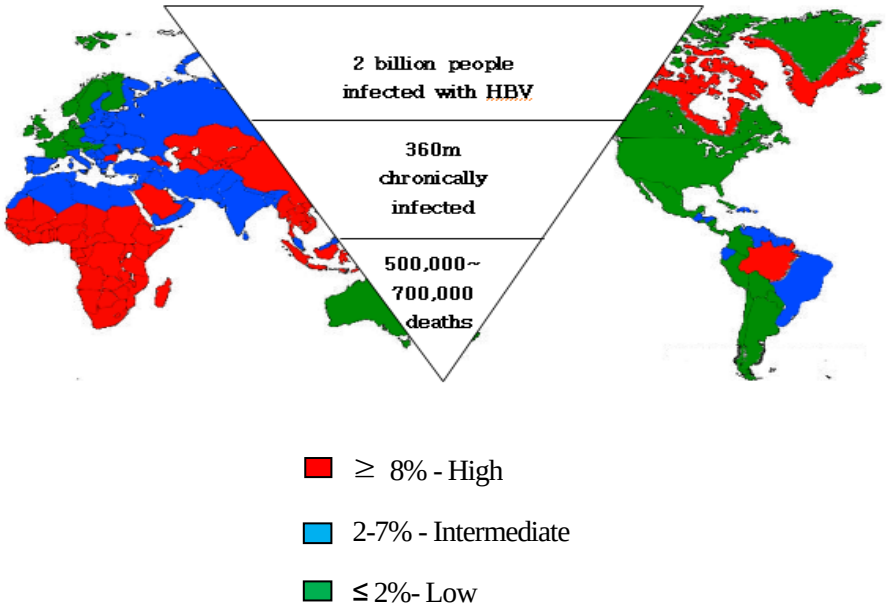


Fig.3. HBV prevalence and mortality

1.1.5 Disease Progression

HBV is a noncytopathic, hepatotropic DNA virus, which causes acute or chronic infection [15,16,17,18,19,20,21,22]. HBV infection is a dynamic process that includes replicative and non-replicative phases ranging from silent, acute phase to persistent chronic stage [23]. Acute HBV infection is normally asymptomatic, but chronic infection continues for several decades, developing to severe and life-threatening complications, such as cirrhosis, liver cancer and hepatic decompensation. Immunity and age are the predominant factors that affect the progression and outcome of such conditions. Adults recover from HBV infection in approximately 95% of the cases, but the recovery rate is low among infants, resulting in chronic or acute hepatitis in other cases. Chronic hepatitis often develops to cirrhosis or liver cancer over the course of 30-50 years until death [23].

1.2 Therapeutic DNA Vaccine

1.2.1 Why is Therapeutic DNA Vaccine Needed?

Currently approved and clinically available therapeutics include Lamivudine, Adeforvir, Entecavir, Telbibvdine, Clevudine and Interferon-α. Lamivudine (Epivir-HBV; GlaxoSmithKline), a nucleoside analogue, is an inhibitor of HBV polymerase. It is effective in inhibiting virus replication during the treatment period but, once the treatment is discontinued, the virus replication starts again, followed by liver damage. Therefore, lamivudine is effective only temporarily and only in about 20%. The occurrence of mutation during lamivudine treatment is another impediment to its long-term use [24]. Other inhibitors of HBV polymerase include Adefovir and Entecavir, which also inhibit HBV polymerase and is capable of inhibiting lamivudine-resistant HBV more effectively [25]. These chemical drugs are inherently limited by the recurrence of serum HBV DNA

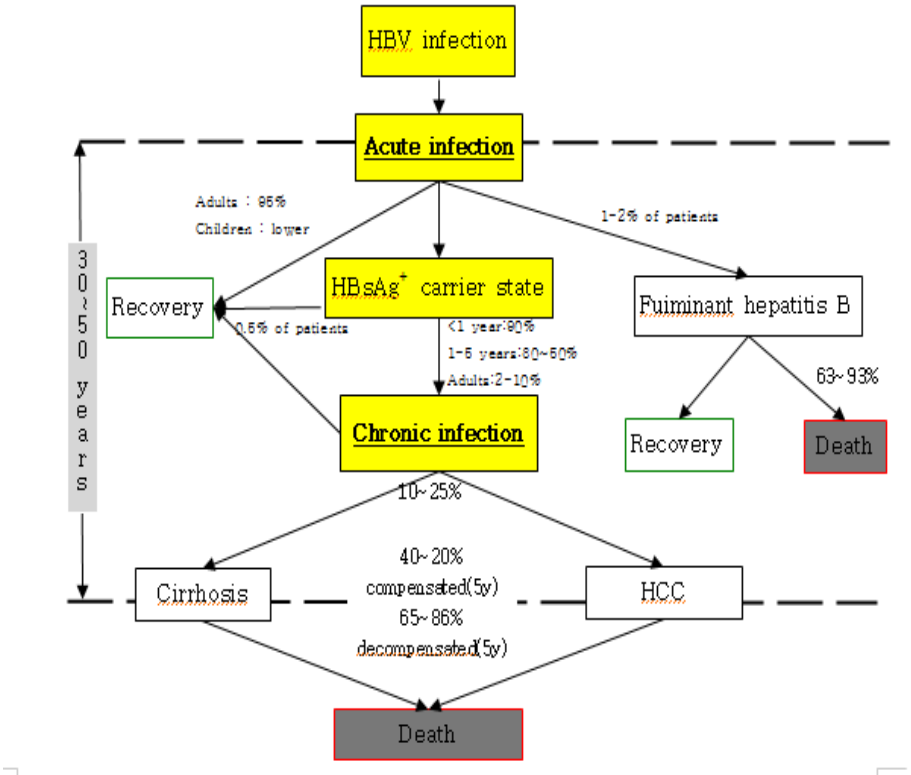


Fig.5. HBV – course of disease. Immunocompetent adults mostly (≥95%) recover from HBV infection, without a risk of chronic infection, but children with a weak immune system often end up being a HBs Ag carrier and develop chronic infection in more than 90% of the cases, resulting in cirrhosis and liver cancer, and ultimately in death, in about 30-50 years.

and ALT levels in most patients after discontinuing the administration. Interferon-alpha inhibits HBV replication by increasing the host immunity but it develops a serious side effects and the therapeutic effect is 25-30% in patients [26, 27, 28].

Based on these side effects and therapeutic limitations of existing therapies in patients with chronic HBV infection (Table 1), this study was aimed at developing a therapeutic vaccine with superior efficacy, which can induce more effective cellular immune response than HBV therapeutics in patients with HBV infection.

1.2.2 Possibility for the Development of Therapeutic Vaccine

Recent clinical studies have found that more potent, multiple allergen-specific CD4 T cell-mediated immune reaction [29] and CD8 Tcell-mediated immune reaction [30, 31] are needed for the recovery of the patients with chronic hepatitis B. Multiple antigen-specific, continuous CD4 and CD8 T cell responses were observed among the patients who have spontaneously recovered from hepatitis B, while antigen-specific CD4+ and CD8+ T cells were induced only for some of the HBV epitopes and detected only temporarily and weakly among the patients with chronic hepatitis B. In addition, these T cells were reported as having low proliferation and cytokine production capacities. Spontaneous recovery occurs in about 1-2% of the patients with chronic hepatitis B. In this case, cell-mediated immunity was observed before HBsAg seroconversion, supporting the importance of cell-mediated immunity for the treatment of chronic hepatitis B. We concluded from the previous results that inducing extensive and potent cell-mediated immunity is very important for the treatment of chronic hepatitis B.

Recombinant protein vaccine using surface antigen was first attempted for the treatment of hepatitis B. The vaccinated group seemed to have been affected by the vaccine in terms of HBV DNA loss and seroconversion in the results of first 6 months. After 1 year of

Table 1. HBV therapeutics and limitation

	IFN-a	**Lamivudine**	**Adefovir**	**Entecavir**
Efficacy				
HBeAg loss	33%	17-32%	24%	16-23%
HBsAg loss	8%	<1%	<1%	1.4%
Duration of Treatment				
HBeAg+	4-6 months	≥1 year	≥1 year	
HBeAg-	1 year	≥1 year	≥1 year	
Route	Subcutaneous	Oral	Oral	Oral
Side effects	Many	Negligible	Nephrotoxicity?	Negligible
Drug resistance	-	~30% (year 1) ~70% (year 5)	none (year 1) ~ 3% (year 2)	
Cost/yr	High	Low	Intermediate	Intermediate

vaccination, however, no difference was observed between the vaccinated group and placebo group.

To overcome the limitations of recombinant protein therapeutic vaccine, DNA vaccine encoding HBV antigen is currently being attempted as well. Once inside the body, the DNA expresses antigen, which undergoes both endogenous and exogenous processes, loaded to both MHC class I and II and induces CD4 and CD8 T cell responses simultaneously. When this gene encoding surface antigen was administered to healthy people, antibody response and cell-mediated immune reaction were both induced without any particular side effects. When CHB patients were vaccinated with DNA that encodes envelope protein (HBV PreS2-S), however, the loss of HBV DNA was detected in only 1 of 10 patients [32]. T cell proliferation was induced in 2 patients, but the response was temporary and low. In other words, immune response could be induced in some patients, but patients would benefit more from a therapeutic vaccine which can induce more potent and effective cell-mediated immune response.

1.2.3 Antigen Gene Selection

Selecting an appropriate antigen gene is important in developing a therapeutic DNA vaccine of HBV. Prophylactic vaccine mostly uses s antigen, but which antigen is effective for therapeutic DNA vaccine is unknown yet. It has been suggested, however, that multivalent antigens should be used because clinical studies constantly failed in developing therapeutic HBV vaccine when they used single epitope or single antigen. In order to determine the necessity of multivalent antigens, immune reaction to HBV antigens was investigated in patients who were spontaneously cured of chronic HBV infection. The patients showed specific cellular immune responses to multi antigens [29]. An immune reaction analysis involving patients in remission after acute infection showed multi-specific

CTL responses [30, 31].

Based on the results in literature, we decided to use multiple genes for HBV therapeutic DNA vaccine development.

1.3 Current Development Status of Therapeutic Vaccine

No therapeutic vaccine for CHB has been released yet, with phase 1 or 2 clinical studies still in the early development phase. Most CHB therapeutic vaccines are in the form of protein or DNA. The development status is summarized in Table 2.

1.4 Preliminary Study: HB-100 (Lead) study

Before developing the candidate (HB-110), preclinical and pilot study were conducted on the lead (HB-100). HB-100 vaccine components and the clinical protocol for the pilot study are presented in Fig. 5.

HB-100 is a multi-DNA vaccine that contains PreS1, PreS2, S, pol and core genes to induce extensive immune response. Newly developed mutant human IL-12 expression vector was used to facilitate CTL and Th1 responses [34]. These HB-100 vectors were transformed into *E. coli* to make production cell line respectively, which was used in a host to produce each expression plasmid and the HB-100 plasmids were produced through multiple purification processes. This HB-100 was found safe in animal in a preclinical study. Induction of HBV-specific immunity was confirmed after DNA vaccination in mice. Phase 1 clinical trial was conducted in 12 CHB patients in Ukraine and Lithuania to identify the safety and efficacy of HB-100 in human. As presented in Fig. 5, a chemical drug Lamivudine was first administered for 4 weeks, and then HB-100 was administered 12 times at one month intervals, in addition to Lamivudine, to the patients whose virus level has decreased more than 10 times. After completing HB-100 administration,

Table 2. Current status of clinical trials on HBV therapeutic vaccine

Drug	Status	Company	Target
HBV infection vaccine, epsilon PA-44	Phase 2	Chongqing Jiachen Biotechnology Ltd	N/A
Hepatitis B virus vaccine (oral, spi-VEC)	Phase 2	Emergent Biosolutions	HBcAg
HBsAg-HBIG complex	Phase 3	Shanghai Medical University	HbsAg complexed human anti-HBs immunoglobulin(HBIg), adjuvanted by alum[33]
Therapeutic HBV vaccine DV-601	Phase 1	Dynavax Technology Corp	HBsAg, HBcAg
DNA vaccine (HBV)	Phase 1	Institut Pasteur	N/A
HB-110	Phase 1	Pohang university of Science & Technology, Dong-A and Genexine	Plasmid DNA vaccine encoding hepatitis B virus protein and mutant IL-12
Hepatitis therapeutic vaccine	Phase 1	Vaxine Pty Ltd	PreS HBsAg, with or without HBcAg

Hepatitis B DNA vaccine	Discovery	Avanti Therapeutics	N/A
HBVax (SimVec, HBV infection), GeneCure	Discovery	Genecure LLC	N/A
Therapeutic hepatitis B vaccine	Discovery	Transgene	N/A
HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; IL = interleukin; N/A = not available			

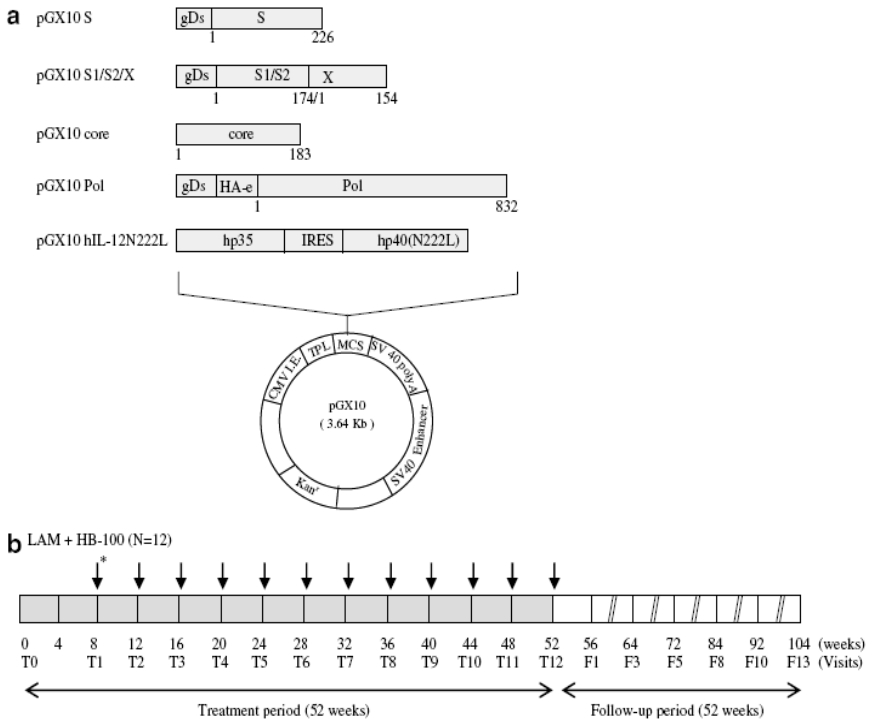


Fig.5. Schematic diagram of the HB-100 vaccine components and the clinical protocol. HB-110 consists of five different plasmids(8mg) including pGX10 S(2mg)+pGX10 S1/S2/X(1.5mg)+pGX10 core(1.5mg)+pGX10 Pol(1mg)+pGX10hIL12N222L, which were based on the pGX10 vector. pGX10 S plasmid vector expresses S antigen gene, pGX10 S1/S2/X plasmid vector expresses pre S1, pre S2 and X genes, pGX10 core expresses core gene, pGX10 Pol plasmid vector expresses polymerase, and pGX10hIL12N222L plasmid vector expresses mutant IL-12.

Lamivudine was discontinued as well, and the subjects were followed for 1 year. HB-100 was related to mild side effects, such as fatigue and headache, but serious adverse events were not observed (Table 3). At the end of the clinical trial, e antigen was not detected in 3 patients among 6 e antigen-positive patients, and e antigen seroconversion was observed. S antigen loss was confirmed in 1 patient. Viral rebound suppression and ALT normalization were observed in 6 out of the 12 patients (Table 4, Fig. 6). These results support the safety and efficacy of HB-100 in terms of e antigen seroconversion and viral rebound suppression. Cell-mediated immunity to env, core and polymerase antigens was increased in the virological responder group to HB-100 [35].

1.5 Selection of Therapeutic DNA Vaccine Candidate (HB-110) and Development System

1.5.1 Candidate (HB-110) Selection

We confirmed the possibility of developing the therapeutic DNA vaccine for CHB patients in the HB-100 study. Codon-optimized HB-110 therapeutic DNA vaccine was developed to enhance its efficacy. The antigen expressions were increased by about 1.5-3 times depending on the genes. The codon-optimized HB-110 therapeutic DNA vaccine contained two types of plasmid DNA (pGX10-S/L, pGX10-C/P) encoding the S, L, C, and P HBV antigens and one plasmid DNA (pGX10-hIL-12m) encoding human IL-12 N222L as a genetic adjuvant to enhance the cellular immune response [34]. The eukaryotic expression vector pGX10, used as a backbone for cloning the HBV genes, harbors a prokaryotic origin of replication (ColE1), a bacterial kanamycin resistance gene, and a eukaryotic expression cassette consisting of a human cytomegalovirus (CMV) promoter, an adenovirus tripartite leader sequence, an SV40 late poly A, and an SV40 enhancer

Table 3. Safety of HB-100

Adverse events	During treatment (N=12)	After treatment (N=12)
Fatigue	2(17%)	3(25%)
Nausea	2(17%)	2(17%)
Abdominal discomfort	2(17%)	2(17%)
Fever	1(8%)	1(8%)
Headache	1(8%)	1(8%)
Sleep disorder	0	0
ALT flare	2(17%)	4(33.3%)
Rash in injection sites	0	0
Pain in injection sites	1(8%)	0
Reaction in injection sites	0	0
Anti-dsDNA antibody	0	0
Anti-nuclear antibody	0	0
Anti-human IL-12N22L antibody	0	0

Table 4. Efficacy of HB-100

Item	End of treatment (LAM+12 vaccinations: 52 weeks)	Follow-up (52-week follow-up after discontinuation of administration)
Loss of HBeAg	4/6	3/6
eAg seroconversion	4/6	3/6
Loss of HBsAg	0/12	1/12
Suppression of viral rebound	10/12	6/12
- Virological responder	10	6
- non-virological responder	2	6
Normalization of ALT	8/12	6/12

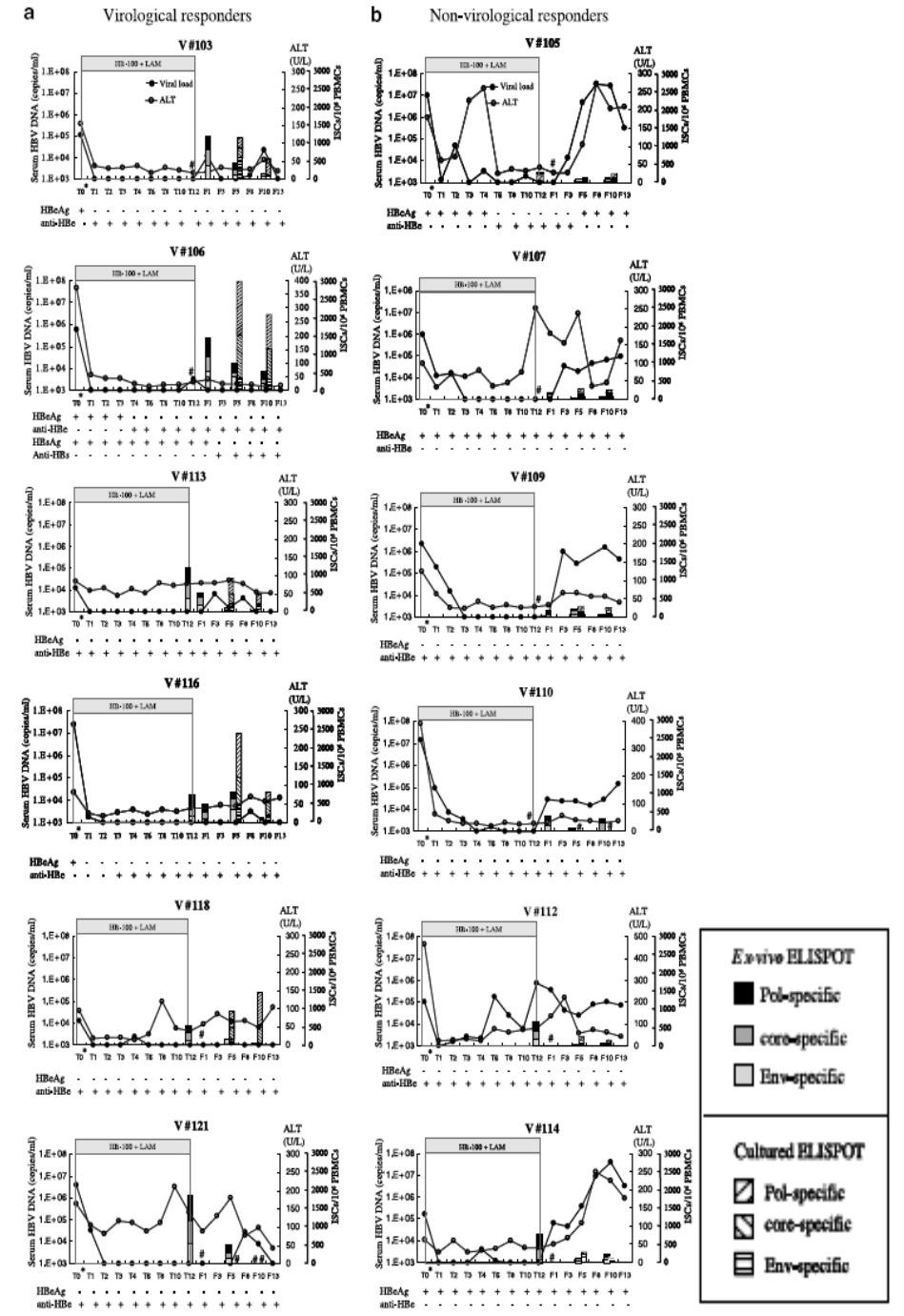


Fig.6. The levels of viral load, ALT, HBV antigen-specific T-cell responses, HBeAg, and HBsAg seroconversion in the VRs (**a**) and in the NVRs (**b**). in the HB-100 clinical trial

(Fig.7). Each plasmid was generated in *Escherichia coli* DH5α cells. pGX10-S/L consists of 7230 base pairs (bp) encoding the envelope proteins (S, L) of 227 amino acids (aa); pGX10-C/P consists of 8653 bp encoding the core protein of 184 aa and polymerase protein of 839 aa; pGX10-hIL-12m consists of 6095 bp encoding the p35 (253 aa) and p40 (328 aa) subunit proteins of the mutant form of human IL-12 (Fig.7.). pGX10-S/L and pGX10-C/P plasmid vectors express antigen genes, such as S, L, core and Pol genes, to induce cellular and humoral immune responses, while pGX10-hIL-12m plasmid vector increases the induced cellular immune response to further enhance the efficacy. HB-110 was formulated in 150 mM sodium–phosphate buffer (pH 7.0) at a 2:1:1 pGX10-S/L: pGX10-C/P: pGX10-hIL-12m ratio.

1.5.2 Development System

Multiple institutions participated in the development of HB-110, as a project(A040013) of the Ministry of Health & Welfare. Dong-A Pharm., as a central organization, conducted the preclinical and clinical studies of HB-110, and efficacy evaluation and immunological analysis in the disease model was performed as the secondary project in Pohang University of Science and Technology (POSTECH). Multiple institutions, including Korea Institute of Toxicology and The Catholic University of Korea Seoul St. Mary's Hospital, participated in toxicity and clinical studies.

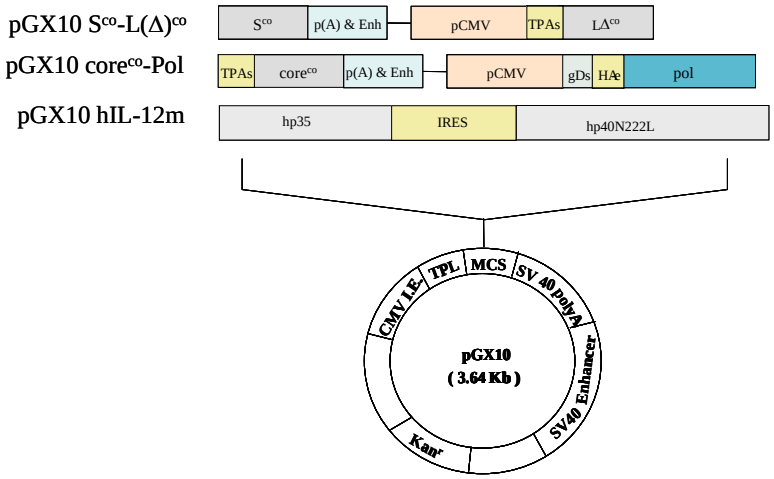


Fig.7. Schematic diagram of the HB-110 vaccine components. The codon-optimized HB-110 therapeutic DNA vaccine contained two types of plasmid DNA (pGX10-S/L, pGX10-C/P) encoding the S, L, C, and P HBV antigens and one plasmid DNA (pGX10-hIL-12m) encoding human IL-12 N222L. pGX10-S/L consists of 7230 base pairs (bp) encoding the envelope proteins (S, L) of 227 amino acids (aa); pGX10-C/P consists of 8653 bp encoding the core protein of 184 aa and polymerase protein of 839 aa; pGX10-hIL-12m consists of 6095 bp encoding the p35 (253 aa) and p40 (328 aa) subunit proteins of the mutant form of human IL-12.

II. MATERIALS AND METHODS

2.1. Establishment of the Production Process for HB-110 Material

For the production of preclinical and clinical material of HB-110, production cell lines were made and the production process was established by developing the fermentation and purification processes.

2.1.1 Selection of Host Cell and Cell Line Manufacturing

The selection of a host cell for the production of naked plasmid DNA is very important, and *E. coli* is most often used for the purpose among other host cells. For the production of plasmid DNA for gene therapy, the host cell should be nonpathogenic, the genotype should be well established, and the phenotype related to the strain should be easily analyzed. The transformed plasmid DNA should have almost zero occurrence of mutation, and the strain should be the one that's commonly used as a host cell for gene therapy. After a review of various literature and data, *E. coli* DH5α (TaKaRa, Cat. No. 9057; Lot No. K198) was considered and selected as the most appropriate host cell for the production of naked plasmid DNA [36]. Production cell line was made by transforming each of 3 plasmid DNAs(HB-110) into *E. coli* DH5α, and plasmid DNA was isolated from the production cell line, which was identified by using agarose gel electrophoresis.

Master cell bank (MCB) and working cell bank (WCB) were established each for pGX10 S/L, pGX10 C/P and pGX10 hIL-12m. Stability, identity and purity of the cell lines were analyzed.

2.1.2 Fermentation Process

Since plasmid production from WCB has great differences in plasmid productivity and quality depending on the condition of the culture medium, it is important to select the optimal culture medium. In this context, WCB were cultured in different types of culture media and their growth rate, plasmid copy number, supercoil shape and plasmid ratio were compared to select the most optimal culture medium. Cell growth rate and plasmid DNA production yield per unit cell were evaluated for various defined and complex media.

The optimal culture condition was determined by testing different conditions with a 30L incubator (Bioengineering NFL 30L). For the fermentation of WCB for plasmid DNA production, the temperature was maintained at 37℃, with 600 rpm stirring, 10 L per minute air inflow, and pH 7.0. Fed-batch process was attempted to increase the productivity of cell line. Glycerol was used as the carbon source and yeast extract as the nitrogen source for additional medium, which was fed when cell growth was decreased and pH started elevating. The fermentation was continued until when it turned from log phase to stationary phase. The cultured broth was then centrifuged for 10 minutes at 3,000 x g and collected.

2.1.3 Purification Process

For isolation and purification of plasmid DNA from the cultured cell, a number of literature on plasmid DNA purification were investigated and a purification process appropriate for the drug specification was selected [37, 38]. The purification process consists of centrifugation of the culture solution of WCB, alkaline lysis, ethanol precipitation, PEG purification, anion exchange chromatography and thiophilic/aromatic adsorption chromatography. The process was optimized for each plasmid by differentiating the condition of elution and fractionation.

2.2 CMC (chemistry, manufacturing & control) Study

Physicochemical and biological characterization was performed using HB-110 produced according to the established process.

2.2.1 Physicochemical Characterization

For physicochemical characterization of the 3 types of plasmids comprising HB-110, sequence, base composition and restriction fragment, molecular conformation on HPLC, spectroscopic character and plasmid size were analyzed. Nucleic acid sequence and composition were confirmed by sequencing of each plasmid, and nucleic acid structure was confirmed by restriction fragment analysis. Molecular conformation was analyzed by using AE-HPLC, and spectroscopic character was analyzed by measuring the absorbance with spectrophotometer. The size of each plasmid was confirmed by electrophoresis after making a single cut with the restriction enzyme.

2.2.2 Biological Characterization

After in vitro transfection for each of the 3 types of plasmid comprising HB-110, Western blot was used for the identification of expressed proteins, RT-PCR for mRNA expression, ELISA for quantification of protein expression, and INF-γ ELISpot assay following administration in mice for biological activity (immunogenicity). COS-7(ATCC#:CRL-1651) and C2C12(ATCC#:CRL-1772) were used as the cell lines. Immunostimulatory effect of IL-12 was confirmed by ELISpot following administration in mice.

2.3. Specifications and Test Procedures

2.3.1 Quality Control Items and Specifications for Plasmid DNA

For the production of plasmid DNA appropriate for medicine, the quality control items and specifications of plasmid were determined to an internationally acceptable level [37, 39], and test methods were established accordingly. Test items include appearance, pH, purity, identification, titer, sterility, bacterial endotoxin, abnormal toxicity, residual kanamycin and ethanol tests. The purity test involved spectroscopic test, molecular conformation test and analyses on host cell-derived peptide, DNA and RNA. The identification test involved DNA sequencing, restriction fragment test and plasmid size analysis.

2.3.2 Establishment and Validation of HBcAg Quantification

Among the HB-110 titer testing, quantitative analysis of *in vitro* HBcAg expression using HBeAg ELISA kit was established, and validation study was conducted to test its suitability. For the quantitative analysis of *in vitro* HBcAg expression, HB-110 was transfected to mouse muscle cell line C2C12, and the quantity of HBcAg released to the culture medium was quantified with the HBeAg ELISA kit. ETI-EBK PLUS (HBeAg ELISA, DiaSorin) was used for the ELISA kit, and HBcAg of KOMA (Cat # : K0121147) was used as the reference material of HBcAg. Lipofectamine PLUS (Invitrogen) was also used as the transfection reagent.

2.3.3 Identification of the Expression of L and Polymerase Proteins

Among HB-110 expression test, Western blotting was established to identify *in vitro* expression of L protein and polymerase gene. For the *in vitro* identification test of

expression, HB-110 was transfected to COS7, after which the cells were collected, lysed, and Western blotting was performed after SDS-PAGE. The antibodies used in this Western blotting are summarized in Table 5.

2.4 Selection of Dosage Form and Stability Test

2.4.1 Dosage Form Design and Long-term Stability Test

For the selection of the dosage form for HB-110, expression rate evaluation was performed using different types of buffer solution to select the primary dosage form. The selected buffer solution was 150 mM sodium phosphate (pH 7.0), with plasmid DNA concentration of 2 mg/ml. The composition rate of the 3 plasmids was 2:1:1 for pGX10-S/L, pGX10-C/P and pGX10-hIL-12m, respectively.

For a long-term safety study, 3 lots of HB-110 samples, each consisting of 150 vials, were manufactured using glass vials as the container. The samples were refrigerated (2-8℃) in a dark place, and tests were performed at intervals of 0, 3, 6, 9, 12 and 18 months. This long-term safety study was conducted in accordance with the KFDA guidelines for long-term safety studies. Table 6 shows the dates when each lot was tested.

The long-term stability test involved appearance, pH, restriction enzyme DNA fragment identification test, purification test (spectroscopic test, molecular conformation test), titer test, sterility test, bacterial endotoxin test, abnormal toxicity test and content test. Host cell-derived peptide, DNA and RNA tests, pyrogen test, foreign insoluble matter test and test for the volume of injection in container were omitted from the long-term test for clinical approval, since these items are not expected to change with time after the production of HB-110.

Table 5. Antibodies used for the identification of the expression of L and polymerase proteins

Antigen	Primary antibody	Secondary antibody
L protein	Mouse anti-HBsAg mAb (Biomeda, cat.# V1060)	Goat anti-mouse IgG alkaline phosphatase conjugate (Sigma, cat.# A-3438)
Polymerase	Rabbit anti-HA pAb (Santa Cruz, cat.# sc-805)	Goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, cat.# A-3687)

Table 6. Sample lots and test date of HB-110 long-term stability test

Lot	HB0505	HB0506	HB0507
Test start date	Mar. 22, 2005	Apr. 07, 2005	Apr. 26, 2005
3 months	Jun. 22, 2005	Jul. 07, 2005	Jul. 26, 2005
6 months	Sep. 22, 2005	Oct. 07, 2005	Oct. 26, 2005
9 months	Dec. 22, 2005	Jan. 07, 2006	Jan. 26, 2006
12 months	Mar. 22, 2006	Apr. 07, 2006	Apr. 26, 2006
18 months	Sep. 22, 2006	Oct. 09, 2006	Oct. 26, 2006

2.5 Safety Evaluation

2.5.1 HB-110 Production for Safety Evaluation

HB-110 required for the safety study in animals were manufactured according to the established process, and quality control tests were conducted before sending it to toxicology institutions (Korea institute of toxicology [KIT], Ina in Japan). Three lots of samples were manufactured for the safety evaluation: the first lot (1.5 g) was used for toxicity test in rats at KIT and the second and third lots for repeated dose toxicity test at Ina.

Quality control test of safety material was performed in accordance with HB-110 specifications and test procedures to determine whether the samples meet the quality standards for the safety samples manufactured according to the production process.

2.5.2 Safety Assessment in Small Animals

HB-110 safety evaluation was performed in rats and mice in KIT, affiliated with Korea Research Institute of Chemical Technology.

2.5.2.1 Acute Toxicity Test for Single Intramuscular Injection in Rats

HB-110 0, 0.25, 1 and 4mg/kg were administered in rats for acute toxicity test. SD rats of around 6 weeks of age were used, 6 of them in each group. Single dose of HB-110 was administered intramuscularly before conducting a 2-week follow-up.

2.5.2.2 Acute Toxicity Test for Single Intravenous Injection in Rats

HB-110 0, 0.25, 1 and 4 mg/kg were administered in rats for acute toxicity test. HB-110 was administered intravenously to SD rats, which were separated by sex. Single dose was administered to 6 rats in each group before conducting a toxicity follow-up for 2 weeks.

2.5.2.3 26-Week Chronic Toxicity Test for Intramuscular Injection in Rats

HB-110 0, 0.25, 1 and 4 mg/kg/1 ml were administered for 26 weeks in rats for chronic repeated intramuscular injection. HB-110 was administered once a week intramuscularly to SD rats, which were around 6 weeks of age. Rats were seperated by sex, and toxicity follow-up was conducted after 26 weeks of administration.

2.5.2.4 Genetic and Reproductive Toxicity Test for HB-110 in Rats

HB-110 0, 0.1 and 0.4 mg/head (50 times the anticipated clinical dose) were intramuscularly administered repeatedly for 5 weeks for genetic and reproductive toxicity tests. ICR mice of around 6 weeks of age were used, 6 of them composing each group. HB-110 was administered once a week intramuscularly, and toxicity follow-up was conducted after 5 times of administration.

2.4.2.5 Immunotoxicity Test for HB-110 in Mice

HB-110 0, 100, 400 (400 times the anticipated clinical dose) ug/100ul/head were intramuscularly administered 4 times every 2 weeks for immunotoxicity test. Female BALB/c mice of 5 weeks of age were used, and immunotoxicity was observed after 4 times of administration at 2-week intervals. OVA was administered to a positive control group.

2.4.3 Safety Evaluation in Monkeys

HB-110 repeated dose toxicity test was performed in non-human primate (CRO, INA Research in Japan). HB-110 was administered biweekly for 6 months (total 13 doses). HB-110 0, 1 or 4 mg/kg was administered intramuscularly to 3 male and female monkeys in

each group (Table 7).

Weight change, general symptoms, food consumption, ocular examination, urinalysis, hematology, serum chemistry, autopsy, organ weight and histopathology were evaluated. For immunogenicity analysis, HBV specific immunogenicity was evaluated by isolating serum at baseline, 10 weeks, 20 weeks and 6 months.

2.6 Pharmacokinetic Study

To investigate the pharmacokinetics of HB-110, serum half-life and AUC, DNA migration into the blood, distribution to tissue and elimination after intramuscular administration were evaluated [40].

2.6.1 HB-110 Preparation

The material for the pharmacokinetic study was manufactured in accordance with the established production process of HB-110. The test was performed after confirming the suitability of the samples.

2.6.2 Intravenous Administration of HB-110

HB-110 was administered to 5-week-old male Balb/C mice as a single injection of 100 μl volume (100 μg total plasmid DNA) via the tail vein. The control mice received 100 μl of 150 mM phosphate buffer, pH 7.0. Five mice were sacrificed at each time point for blood and tissue analysis. Blood samples were drawn from the retro-orbital plexus at 1, 5, 15, 30, 45, and 60 min post-injection in the presence of heparin. The samples were stored at -70℃ and analyzed within one week.

Table 7. HB-110 repeated dose toxicity in non-human primate

Group	Substance	Dose level (mg/kg/day)	Concentration (mg/ml)	Dose volume (ml/kg)
Control	Sodium phosphate buffer	0	-	1
Low	HB-110	1	1	1
High	HB-110	4	4	1

Group	Number of animals									
	Total		Clinical laboratory				Terminal necropsy		Histopathology	
			Pretest		Term					
	M	F	M	F	M	F	M	F	M	F
Control	3	3	3	3	3	3	3	3	3	3
Low	3	3	3	3	3	3	3	3	3	3
High	3	3	3	3	3	3	3	3	3	3

M=Male, F=Female, Term=Termination of dosing

2.6.3 Intramuscular Administration of HB-110

HB-110 was administered as a single 50 μl injection (100 μg total plasmid DNA) into the left tibialis anterior muscle of male Balb/C mice. The control mice received 50 μl of 150 mM phosphate buffer, pH 7.0. Five mice were sacrificed at each time point for analysis. Blood and muscle samples were taken at 5, 15, 30, 90 min, 2 h, 8 h, day 1, day 11, day 14, and day 30 post-administration. Other tissue samples (brain, heart, liver lung kidney, testis, spleen) were taken at 90 min, 8 h, day 1, day 11, day 14, and day 30. All tissue samples were stored at -70℃ until further analysis.

2.6.4 Analysis of HB-110 in Blood Samples by Polymerase Chain Reaction (PCR)

0.7 ㎕ of blood was used directly as a template for PCR using the HemoKlenTaq polymerase PCR kit (DNA polymerase Technology Inc., USA). The primer sequences were 5'-CAGAAGAACTCGTCAAGAAGG-3' and 5'-CATGATTGAACAAGATGGATT-3', which are specific to the kanamycin resistance gene in all three plasmids of HB-110. PCR was carried out using a PTC-200 Peltier Thermal Cycler (MJ Research Co., USA), with 35 cycles of denaturation at 94°C, annealing at 62°C and extension at 68°C, each for 90 seconds. Various dilutions of HB-110 were used as standards. The resulting 795 bp amplicon was verified by 1% agarose gel electrophoresis containing ethidium bromide. Densitometric analysis using a BIO-PROFIL system and BIO-1D Image Analysis software (Vilber Lourmat, France) was used for quantification. Blood from control animals was used as the negative control, and negative control blood spiked with HB-110 was used as the positive control. The PCR analysis had a detection limit of 0.01 pg with a log linear range of 0.1~ 10 ng.

2.6.5 Analysis of HB-110 in Tissue Samples by Polymerase Chain Reaction (PCR)

All tissue samples were weighed, minced with a surgical blade and suspended in 300 ㎕ of lysis buffer (60 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.5% SDS, 400 ㎍/㎖ Proteinase K). After incubating at 55°C for 6 h, DNA was isolated from the supernatant using a commercial DNA purification kit (Promega Co., USA). DNA was eluted in deionized water and stored at -20°C until further analysis. PCR was carried out using the PCR Core system (Promega Co.,USA). The PCR conditions and quantification process were identical to those described for the blood samples.

2.6.6 Evaluation of Biodistribution using RT-PCR

In November 2007, the FDA published a guideline [FDA guideline for plasmid DNA vaccines for infectious disease indications] recommending real-time PCR with detection limit of 100 copy/μg genomic DNA for the analysis of biodistribution in nonclinical trial. Another recommendation is that the residual plasmid DNA should be less than 30000 copy/μg genomic DNA in the injection site at the end of the trial in order to be exempted from plasmid DNA integration test. We established the real-time PCR method with a quantitative detection limit of 100 copy/μg genomic DNA, as recommended by the KFDA, and accurately measured the residual HB-110 in each tissue of rats so as to determine the DNA reduction pattern in tissue after day 1, which was not available in the previous study (2005).

To develop a highly sensitive RT-PCR method as recommended by the FDA guideline and KFDA recommendation, approximately 20 primer-probe sets were screened and the primer and probe that could quantify up to 10 copies of HB-110 were selected.

Using the developed RT-PCR method, HB-110 was administered once or repeatedly

into the tibialis anterior muscular tissue of SD rats and residual plasmid copies per ug gemomic DNA was measured at prespecified intervals for 60 days.

2.7 HB-110 Manufacturing for Phase 1 Clinical Trial

HB-110 for the phase 1 clinical trial was manufactured by Biotech Manufacturing Department in Dalseong Plant of Dong-A Pharm., a GMP-licensed manufacturing facility, as presented in Table 8. After a quality assessment, HB-110 was provided to the clinical trial center, the Catholic University of Korea Seoul St. Mary's Hospital.

2.8 Phase 1 Clinical Trial on HB-110

Based on the data obtained from the preclinical studies, KFDA approved the IND application (safety/efficacy, specifications and test procedures, protocol, etc.) for the phase 1 clinical trial in May 2007. Once obtaining the approval, the Catholic University of Korea Seoul St. Mary's Hospital was commissioned to perform the phase 1 clinical trial, titled 'A 48-week, single-center, open-label, dose titration, randomized phase 1 clinical trial on the safety of intramuscular HB-110 gene therapy combined with oral Adefovir in patients with chronic hepatitis B'. Principal investigator was Dr. Seung-Kew Yoon, Prof. of Gastroenterology. Subjects were CHB patients, aged between 18 and 60, with positive HBeAg at screening (VP1)[41].

2.9 Study of Electroporation Application

Unlike previous chemical drugs, which inhibit the enzymes involved with viral proliferation and control, HB-110 is a candidate therapeutic DNA vaccine that exerts antiviral activity by inducing immune reaction through intracellular expression. The pharmacokinetic study of HB-110 found very low intracellular delivery of plasmid DNA

Table 8. GMP manufacturing of HB-110 for clinical trial

Lot	Date of manufacture	Quantity
VB70901	Sep. 17, 2007	497 vials
VB80702	Jul. 22, 2008	536 vial
Manufacturing facility: Dong-A Pharm. Dalseong Plant (29-40, Bolli-ri, Nongong-eup, Dalseong-gun, Daegu, Korea)		

because it is administered in the form of naked plasmid DNA [24]. In order to increase the intracellular delivery of the naked DNA and enhance its efficacy, eletroporation was applied to HB-110 as follows [42].

2.9.1 HB-110 Preparation

HB-100 manufactured in accordance with the established production process was used in this study after confirming their suitability.

2.9.2 Experimental Animals and Immunization

Male 6-week-old Balb/c mice were received 100 μg of total plasmid DNA (in a volume of 50 μl) on the left tibialis anterior (TA) muscle, immediately followed by electroporation. Two needle array electrodes (BTX ECM 830) were utilized for electroporation. The distance between the electrodes was 5 mm, and the array was inserted longitudinally into the muscle fibers. *In vivo* electroporation was conducted at 100V/cm using a BTX 830 wave generator. 6 unipolar pulses were applied, each lasting for 20 milliseconds. Immunizations were conducted three times at weeks 0, 2, and 4 or twice at weeks 0 and 4 for the evaluation of immune response. Antigen expression was assessed after the administration of a single dose.

2.9.3 Measurement of expressed S Antigen.

Mice were sacrificed on days 2, 5, 10, and 20 after vaccination, and the anterior tibialis muscles were removed and stored in a deep freezer until analysis. 5 mice were used per group. The frozen tissue was homogenized with a OMNI homogenizer (OMNI international, TH-115) in 400ul of lysis buffer (25mM Tris, pH7.4, 50 mM NaCl, 0.5% Na-Doxycholate, 2% NP-40, 0.2% SDS) containing protease inhibitor cocktail (Promega) ,

and was then subjected to an HBs ELISA assay (ETI-MAK-4, N0019, DiaSorin).

2.9.4 Measurement of Antibody Responses

Mice vaccinated twice at 4-week intervals were sacrificed on weeks 3, 4, 6, and 12 after the second immunization. 5 mice were used per group. Antibody responses against HBs antigen and HBc antigen were assessed using anti-HBs ELISA (Murex anti-HBs, C022K95GB, Abbott) and anti-HBc ELISA (Murex anti-HBc, C04GE65GB, Abbott), respectively. The OD value was directly proportional to the quantity of anti-HBs antibodies, but was inversely proportional to the quantity of anti-HBc antibodies.

2.9.5 IFN-r ELISPOT Assay.

Mice vaccinated twice at 4-week intervals or three times at 2-week intervals were sacrificed 3 weeks after the final immunization. 10 mice were used per group. The splenocytes were isolated using ficolle gradients and were subjected to ELISPOT assay using a commercial kit (Mouse IFN-r, EL485, R&D Systems). The numbers of IFN-r secreting cells were counted with a Bioreader-4000 system (Biosys). The splenocytes were stimulated by four peptide pools of S, preS, Core, and Pol.

2.9.6 HBsAg Seroconversion Analysis.

HBV transgenic mice were used which replicate the full HBV genome in the liver. The transgenic mice, originally constructed at Kumamoto University of Japan (Araki et al., 1989), were bred and maintained at Pohang University of Science and Technology. The mice were divided into three groups, receiving either vector control (pGX10), HB-110 or HB-110 with subsequent electroporation. Each group contained three female and three male mice, except for the vector control group, which contained two female and two male

mice. Mice were vaccinated three times at 2-week intervals, each with a dose of 50 ug of HB-110. Electroporation was carried out as described above. Blood was collected at 2 weeks after final vaccination and analyzed for HBsAg seroconversion by HBsAg ELISA and anti-HBs antibody ELISA.

III. RESULTS

3.1. Establishment of HB-110 Manufacturing Process

3.1.1 Cell Line Preparation

Three plasmids of HB-110 were transformed into *E. coli* DH5α respectively and production cell lines were manufactured. MCB and WCB were manufactured each for the production cell line in GMP and the suitability of the cell lines were identified by several studies (Table 9). *E. coli* DH5a was confirmed as the host cell in RAPD method for genotyping of production cell line and as the phenotype in API 20E identification system. The sequence of the plasmid isolated from WCB culture was identical to an expected sequence. Number of plasmid per cell was approximately 130-200 copies/cell. Subculture stability was maintained by 100 subcultures. Cell line purity test confirmed absence of contamination by fungus, other bacteria or bacteriophage.

These results all indicate that the cell line bank was manufactured properly and that it could be used for further preclinical and clinical studies and for production of samples for sale.

3.1.2 Fermentation Process

The fermentation medium containing Yeast extract and Tryptone (Table 10) was selected as the optimal medium for WCB fermentation.

Fermentation condition study was performed in a bioreactor using the selected medium. Cell number could be increased by feeding glycerol and yeast extract at about 7 hours after seeding (Fig. 8). Glycerol was added as a carbon source to the basal medium and feeding medium during the culture process. Glycerol, rather than glucose, was used because it

Table 9. Identification test for HB-110 production cell line

	Test item	Result
Master Cell Bank	Genotype (RAPD analysis)	*E.coli* DH5a identified
	Phenotype (Microbial identification using API 20E)	*E.coli* DH5a identified
	Plasmid DNA uniformity - Sequencing - Restriction fragment identification	 Matched Matched
	Plasmid Yield	Acceptable
	Cell stability	Stable until 100th generation
	Purity - Bacterial and fungal contamination test - Gram-positive contamination test - Bacteriophage contamination test	 Acceptable Acceptable Acceptable
Working Cell Bank	Plasmid DNA uniformity - Sequencing - Restriction fragment identification	 Matched Matched
	Plasmid Yield	Acceptable
	Cell stability	Stable until 100th generation

	Purity - Bacterial and fungal contamination test - Gram-positive contamination test - Bacteriophage contamination test	 Acceptable Acceptable Acceptable
Final Production Cell	Genotype (RAPD analysis)	*E.coli* DH5a identified
	Phenotype (Microbial identification using API 20E)	*E.coli* DH5a identified
	Plasmid DNA uniformity - Sequencing - Restriction fragment identification	 Matched Matched
	Purity - Bacterial and fungal contamination test - Gram-positive contamination test - Bacteriophage contamination test	 Acceptable Acceptable Acceptable

Table 10. Fermentation medium composition of WCB

Component	Concentration(g/L)
Trytone	15 g
Yeast extract	30 g
K2HPO4	9.4 g
KH2PO4	2.2 g
Glycerol	40 ml

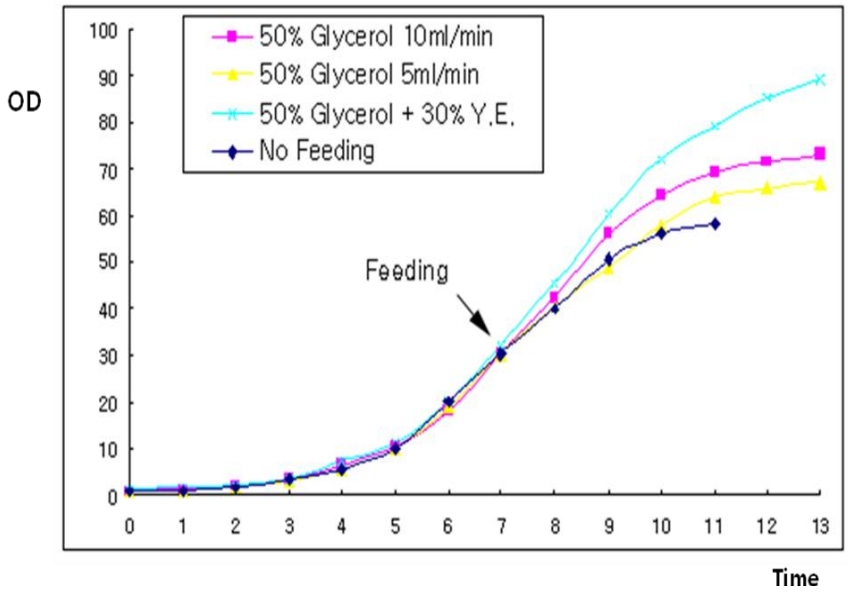


Fig.8. Fed-batch Culturing of HB-110 Producer Cell Line. Different conditions of feeding media were added in the course of culturing HB-110 producer cell line to evaluate growth rate of the cell line. 30% yeast extract containing 50% glycerol was found as the most appropriate one.

tends to increase supercoiled monomer content, presumably because it delays cell growth rate compared to when glucose was added, reducing the rate of multimer production and increasing the content of monomer.

Cell concentration reached OD 90 through the culture process, and approximately 1kg of cells (moist weight) could be obtained per fermentation, which is appropriate for manufacturing preclinical and clinical samples.

3. 1. 3 Purification Process

The final HB-110 purification process was established by performing additional process study to the previous HB-100 production process. A new chromatography was introduced to improve monomer content.

3.1.3.1 Thiophilic/Aromatic Absorption (TAA) Chromatography

Thiophilic/aromatic absorption (TAA) chromatography was introduced to remove open circular form (oc) and other isoform from plasmid DNA, so that the content of supercoiled plasmid DNA could be increased, and to further eliminate residual impurities, such as proteins and endotoxins, from the previous step (Fig.9).

3.1.3.2 Overall Manufacturing Process

Production cell line WCB was used to perform culture and purification process development study and to establish the final HB-110 production process. As can be seen in the Fig.10, the overall production process consists of manufacturing a stock solution through fermentation, purification and concentration from the cell line bank and then final sterile filtration before vialing.

The purity and quality of the manufactured plasmid DNA was enhanced by overall purification study. PEG purification process particularly reduced host chromosomal DNA, which is considered an impurity, and TAA chromatography enabled making

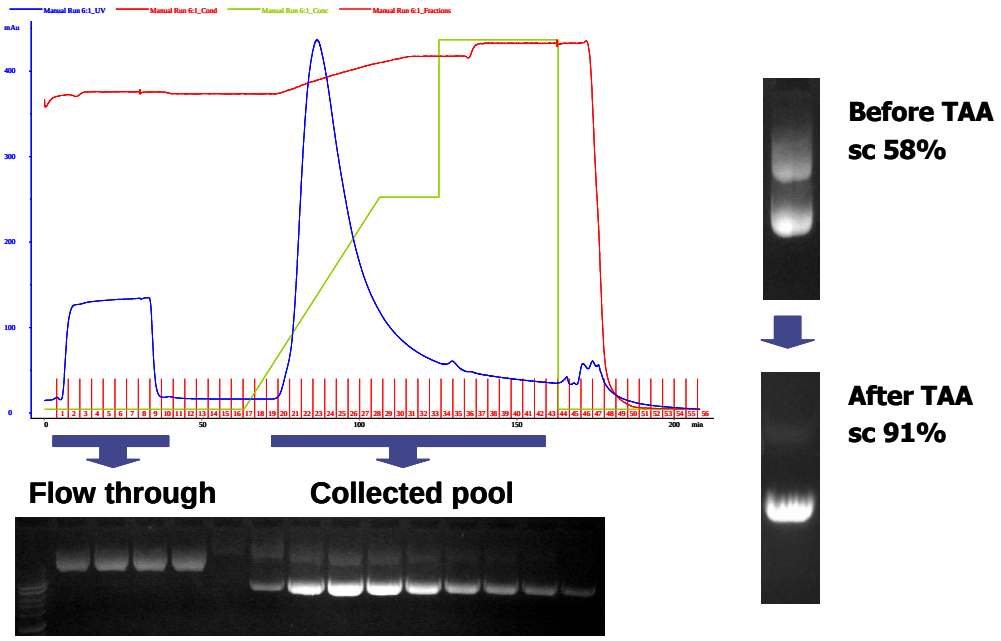


Fig.9. TAA chromatography profile and improvement in sc. Different types of plasmids had different strength of bonding to chromatography resin (functional group, -SH), since nicked or multimer were eluted with low salt and supercoiled monomer with high salt, the content of supercoiled monomer could be increased by TAA chromatography.

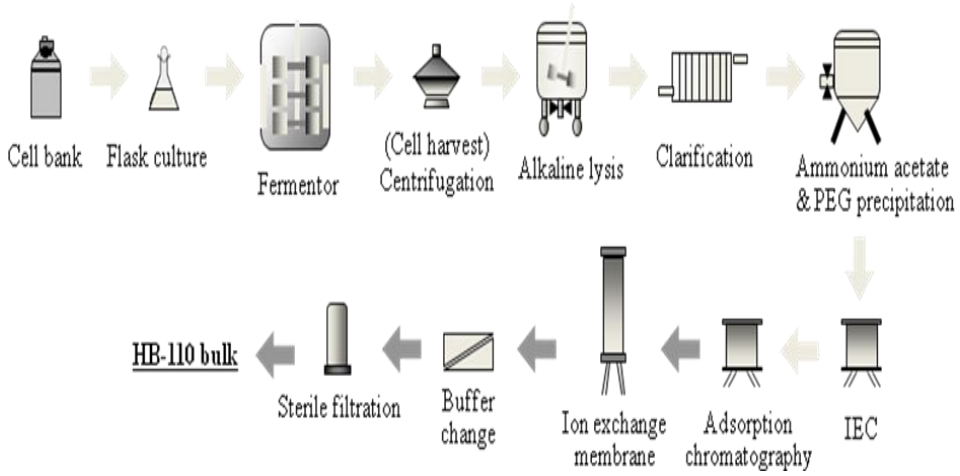


Fig.10. Schematic diagram of HB-110 production process

more than 90% content of supercoiled monomer.

HB-110 sample manufactured by the established manufacturing process was analyzed and the process was found to be eligible for clinical sample production in terms of purity and titer.

3.1.3.3 Yield per Purification Step and In-process Test

Purification was performed using approximately 180 g (moist weight) of pGX10-S/L production cell in accordance with the established HB-110 purification process. The output was found as satisfying the standards for HCP, HCD and endotoxin, and the yield was approximately 28%, suggesting that the process was economically feasible (Table 11). Other 2 types of plasmid production cell lines produced similar results as pGX10-S/L.

3.2 CMC Study

3.2.1 Physicochemical Characterization

After the production of HB-110, its physicochemical characteristics, including the sequence, plasmid structure, UV absorbance and size, were analyzed. Analyses results for required items were confirmed as seen in Table 12.

More specifically, the 3 types of plasmid DNAs were sequenced and found 100% identical to the expected sequence. They were cleaved by using various restriction enzymes for size analysis and were confirmed as present in expected locations. AE-HPLC confirmed that more than 90% were supercoiled monomers in all 3 of them. UV absorbance (A260/A280) was between 1.8 and 2.0.

These results indicate that the 3 types of plasmids in HB-110 were produced properly.

Table 11. Yield per HB-110 purification step

Production Step	Plasmid DNA (mg)	Accumulated yield per step	Host cell-derived peptide (ng/mg)	Host cell-derived DNA (μg/mg)	Bacterial endotoxin (EU/mg)
Bacteria (wet weight: 180g)	266		-	-	-
TFF concentration	266	1	0.3×10^5	62	2.3×10^6
Ammonium acetate precipitation	212	0.80	1.7×10^3	33	1.9×10^4
PEG-8000 precipitation	173	0.65	3.0×10^2	12	6.3×10^3
Anion exchange chromatography	120	0.45	11	5	7
TAA chromatography	90	0.34	0.52	1.5	3.2
Anion exchange membrane chromatography	75	0.28	0.2	1.0	0.9

Table 12. Physicochemical characterization of HB-110

Objective	Method	Result
Nucleic acid structure and composition	Sequencing	Sequence and composition identified
Nucleic acid structure	Restriction fragment analysis	Matches with anticipated fragment
Molecular conformation	AE-HPLC	Present in the form of supercoiled monomeric (>90%)
Spectroscopic profile	UV absorbance spectrum analysis (A260/A280)	1.8-2.0
Plasmid size	Single cut and electrophoresis	Matches with anticipation

3.2.2 Biological Characterization

Biological characteristics of the produced HB-110, including antigen gene expression, quantification of expressed proteins and immune stimulation effect of hIL-12m gene, were analyzed. Protein expression to L antigen, core, pol and hIL-12m was confirmed by Fig. 11. RT-PCR assay confirmed the expression of L antigen and pol gene mRNA(Fig.12). Quantification method for S antigen and c core antigen protein expression was established. The results found that 2.5ng/ml of S antigen and 3.2ng/ml of core antigen were expressed per 1 ug of HB-110.

ELIspot assay was used to confirm immune stimulation effect by hIL-12m. Cellular immune response was increased by 1.7-3 times by adding hIL-12 m gene (Fig. 13).

The biological characterization confirmed intracellular expression of target genes. Quantification method was established for critical core and S antigens and was selected as a QC assessment item for evaluating the eligibility of produced samples.

3.3. Quality Assessment Specifications

3.3.1 Quality Assessment Items and Specifications for HB-110 plasmid DNA

Quality assessment items and specifications are presented in Table 12. Among the main items, the shape of a plasmid was $\geq$90% supercoiled monomer, HCD content was $\leq$ 0.01 mg/mgDNA and pyrogenic endotoxine was $\leq$ 30 EU/mgDNA.

Quality assessment standards, test items and test methods for HB-110 were established based on quality standards for gene therapies using naked plasmids. Clinical samples and samples for product manufacturing will be evaluated for eligibility based on the criteria listed in Table 13.

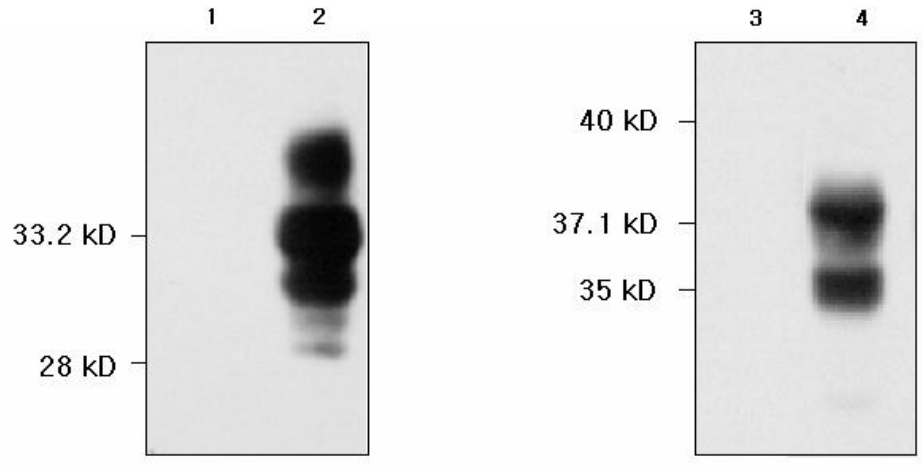


Fig.11. Expression of hIL-12m in COS-7 cell (p35, p40)
Lane 1 : pGX10-transfected cell (Negative control)
Lane 2 : pGX10-hIL-12m -transfected cell (Anti-p35 polyclonal antibody)
Lane 3 : pGX10-transfected cell (Negative control)
Lane 4 : pGX10-hIL-12m (Anti-p40 polyclonal antibody)

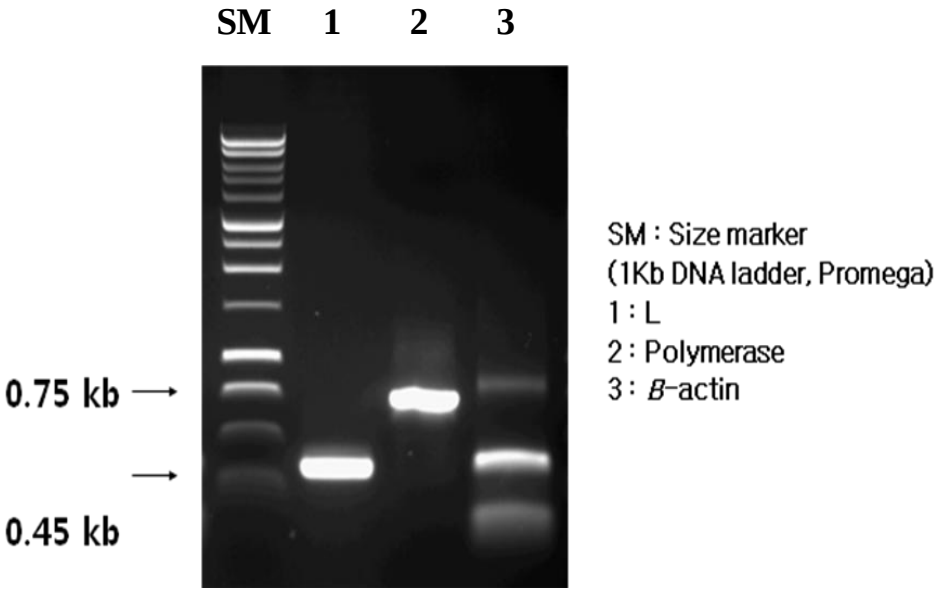


Fig.12. mRNA expression of L and Pol antigens. RT-PCR method was used to confirm mRNA expression of L and Pol antigens, which are antigen genes of HB-110.

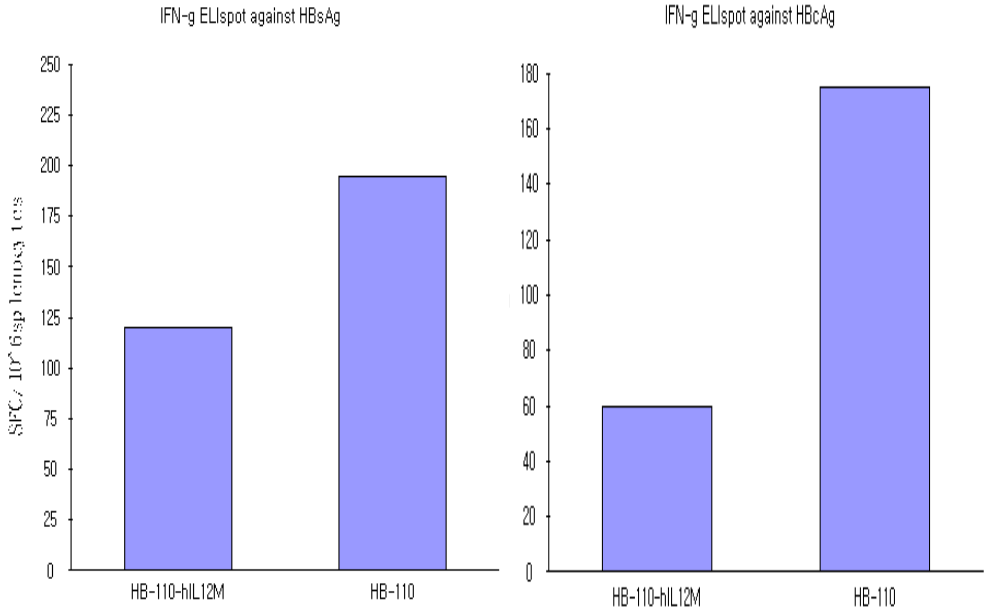


Fig.13. Cellular immune response stimulation by hIL-12m

Table 13. Quality control items and specifications for plasmid DNA

Test item		Normal range
Appearance		Clear colorless solution
pH		6.0-8.0
Purity	Spectroscopic test (A260/A280)	1.8-2.0
	Molecular conformation test	supercoil > 90%
	Host cell-derived peptide test	≤ 3 ng/mgDNA
	Host cell-derived DNA test	≤ 0.01 mg/mgDNA
	Host cell-derived RNA test	Acceptable
Identification	DNA sequencing	100% matched with reference material
	Restriction enzyme DNA fragment identification	Acceptable
	Plasmid size	Matched with reference material
Titer		Expression and immunogenicity confirmed (ELIspot)
Sterility test		Biological product specifications and test procedures (negative)
Bacterial endotoxin test		≤ 30 EU/mgDNA
Abnormal toxicity test		Biological product specifications and test procedures
Residual kanamycin		≤ 5ng/4mgDNA

3.3.2 Establishment and Validation of HBcAg Quantification

Validation tests were performed for the established HBcAg quantification method. Test items included accuracy, precision, quantitation limit, linearity and 3 other types of tests. The results confirmed the acceptability of the quantification method, as can be seen in Table 14. Quantification limit was 5 ng/ml, and linearity was confirmed at R^2 value $\geq$ 0.99.

The HBcAg quantification kit selected for this study was valiadated as appropriate for quantification of HB-110 expression

3.3.3 Identification of the Expression of L and Polymerase Proteins

L antigen and polymerase gene expression were identified by *in vitro* assay as presented in Fig.14. L antigen was identified at about 40 kD and polymerase at about 100 kD, as expected.

3.4 Stability

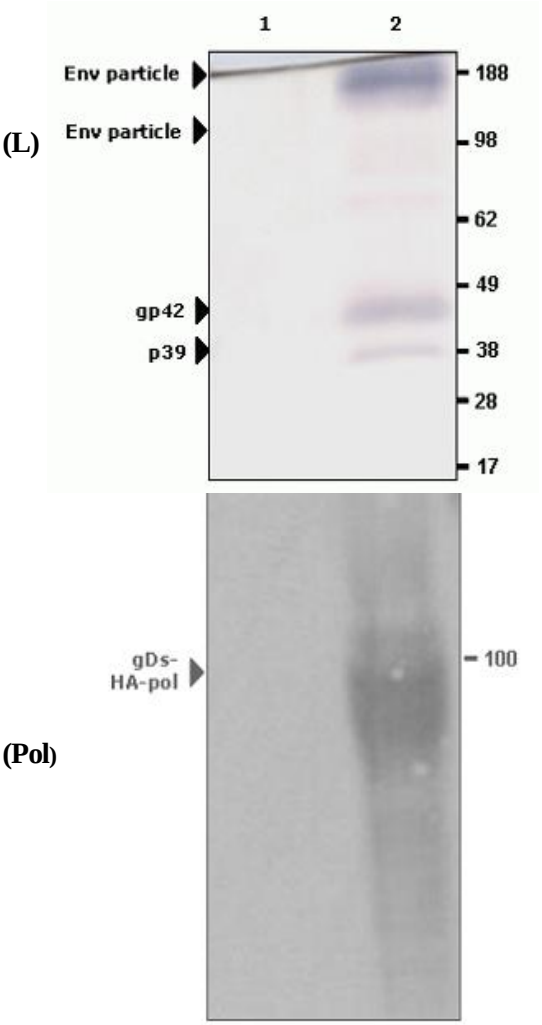
3.4.1 HB-110 Long-term Stability Test

Glass vials containing HB-110 liquid preparation were stored in a dark and cool (2-8℃) place until conducting the stability test at the test start date and 3, 6, 9, 12 and 18 months. Long-term stability test was performed for the items selected in the specifications and test procedures. The results were acceptable for all of the items from the start date to 18 months, suggesting that the quality of HB-110 liquid preparation is guaranteed for 18 months in cold storage (Table 15).

According to the stability test of HB-110, 18-month stability seems appropriate considering future clinical study period and product expiration date.

Table 14. HBcAg quantification validation

Item		Result	Remarks
1. Accuracy		80-120% recovery for all samples	Verified
2.Precision	Repeatability	Standard deviation < 3.00 and CV% < 5.00 when 50 and 100ng/ml reference samples were analyzed on a single ELISA plate	Verified
	Reproducibility	CV% < 20.00 when 6 types of identical samples were 8 repeated tests	Verified
3. Quantitation limit		Quantitation secured after 8 repeated tests with standard solution of 5-200ng/ml concentration	<5ng/ml
4. Linearity		R^2 value ≥ 0.99 after 8 repeated tests with standard solution of 5-200ng/ml concentration	Verified
5. Range		Quantitation and linearity verified by 8 tests with standard solution of 5-200ng/ml concentration	5-200ng/ml
6. Specificity		No interference effect by FBS and other unknown ingredients was found in more than 8 repeated tests.	Verified
7. System suitability		System suitability standards were established for the following items after more than 8 repeated tests: - OD450 values of concentration reference solutions - R^2 value of the regression line for concentration reference solutions - Result value for HB-110 reference	Specifications determined



(L)

Lane 1 : pGX10-transduced cell

(Negative control)

Lane 2 : pGX10-S/L transduced cell

(Pol)

Lane 1 : pGX10- tranduced cell

Lane2: pGX10-C/P cells

Fig.14. L and polymerase antigen gene expression by HB-110. Western blotting confirmed the expression of L(per s1+s2+s) and Pol(polymerase) in antigens of HB-110.

Table 15. HB-110 long-term stability test

Item		Normal range	Result					
			0 month	3 months	6 months	9 months	12 months	18 months
Appearance		Clear colorless solution	OK	OK	OK	OK	OK	OK
pH		6.0~8.0	OK	OK	OK	OK	OK	OK
Restriction enzyme DNA fragment identification		Acceptable	OK	OK	OK	OK	OK	OK
Purity	Spectroscopy	A_{260}/A_{280} = 1.8~2.0	OK	OK	OK	OK	OK	OK
	Molecular conformation	Supercoil ≥ 90%	OK	OK	OK	OK	OK	OK
Titer	HBsAg expression	≥1,000 pg/㎍DNA	OK	OK	OK	OK	OK	OK
	HBcAg expression	≥1,000 pg/㎍DNA	OK	OK	OK	OK	OK	OK
	IL-12 expression	≥100 pg/㎍DNA	OK	OK	OK	OK	OK	OK
	mRNA expression	L/P antigen mRNA identified	OK	OK	OK	OK	OK	OK
Sterility		Acceptable	OK	OK	OK	OK	OK	OK
Bacterial endotoxin		≤30 EU/㎎DNA	OK	OK	OK	OK	OK	OK
Abnormal toxicity		Acceptable	OK	OK	OK	OK	OK	OK
Content		1.8~2.2 ㎎/㎖	OK	OK	OK	OK	OK	OK

3.5 Safety Evaluation

3.5.1 Material for Safety Evaluation

HB-110 three lots were manufactured for safety evaluation, and the established QC method confirmed all the samples as acceptable (Table 16). Among the main evaluation items, supercoiled monomer was 91.5%, HCD was 0.0012 mg/mgDNA and endotoxin was 2.3 EU/mgDNA, suggesting that the safety materials were acceptable.

Three lots of HB-110 samples with confirmed QC eligibility were used for toxicity tests in rats, mice and monkeys.

3.5.2 Safety Assessment in Small Animals and Monkeys

KIT was commissioned to conduct single dose, repeated dose (26 weeks) dose toxicity, genotoxicity, reproductive toxicity and immunotoxicity tests in small animals, such as rats and mice, none of which found any toxicity with maximum dose (4 mg/kg)(Appendix 1). Ina was commissioned to conduct toxicity tests in momkeys. Toxicity was not observed after 26-week repeated dose toxicity test with the maximum dose (4 mg/kg). Anti-HBs antibody was increased in monkeys when the duration and number of administration were increased(Appendix 2).

These results in animals suggest that HB-110 is a safe up to the maximum dose (4 mg/kg), which is approximately 100 times the expected dose in human (4 mg/person). As its safety was confirmed in toxicity tests, it was expected that a clinical study in human was possible.

Table 16. Quality control test of HB-110 safety test material

■ Lot HB0501

Item		Normal range	Result
Appearance		Clear colorless solution	Clear colorless solution
pH		6.0-8.0	7.1
Purity	Spectroscopy (A260/A280)	1.8-2.0	1.915
	Molecular conformation	supercoil > 80%	91.5%
	Host cell-derived peptide	≤ 3 ng/mgDNA	0.3 ng/mgDNA
	Host cell-derived DNA	≤ 0.01 mg/mgDNA	0.0012 mg/mgDNA
	Host cell-derived RNA	Acceptable	Acceptable
Identification	DNA sequence	100% matched with reference material	100% matched with reference material
	Restriction enzyme DNA fragment	Acceptable	Acceptable
	Plasmid size	Matched with reference material	Matched with reference material
Titer		Acceptable	Acceptable
Sterility		Biological product specifications and test procedures	Negative
Bacterial endotoxin		≤ 30 EU/mgDNA	2.30 EU/mgDNA
Abnormal toxicity		Biological product specifications and test procedures	Normal
Residual kanamycin		≤ 5 ng/4 mgDNA	Acceptable
Residual ethanol		≤ 150 ppm	2 ppm

3.6 Pharmacokinetic Study

3.6.1 In Vivo Kinetics of HB-110 after Intravenous Administration

HB-110 was rapidly degraded in blood after intravenous administration (Fig.15.(b)). The half-life of HB-110 in blood was 1.9±0.083 min, and the area under the curve(AUC) was 103±55.81 ug min/㎖. The concentration of HB-110 remaining was < 1% of the initial concentration at 30 min and was not detected 60 min after administration except in one individual with a level at the detection limit (0.01 pg).

3.6. 2 In Vivo Kinetics of HB-110 after Intramuscular Administration

HB-110 remained at the injection site for a considerable time period compared with intravenous administration. PCR analysis of HB-110 showed that approximately 33% and 13% of the initial concentration was present at 15 min and 30 min after i.m. administration, respectively (Fig.16.(a)).

However, there was < 5% remaining 90 min after the injection. Sub-picogram (per mg tissue) quantities of HB-110 were detected for several days. In one individual, plasmid DNA was observed at near quantification limit at day 11 (Table 17).

It was found that intramuscularly administered HB-110 remained for relatively long in muscular tissue where immune reaction could be better induced, while it was lost within a short period of time in other organs. These results suggest that HB-110 should be administered intramuscularly for efficacy and safety in terms of PK.

The blood HB-110 concentration after intramuscular administration showed a maximum of 604 pg/ml at 15 min. However, HB-110 degraded continuously to 24 pg/ml at 30 min and was not detected at 90 min after administration (Fig.16(b)).

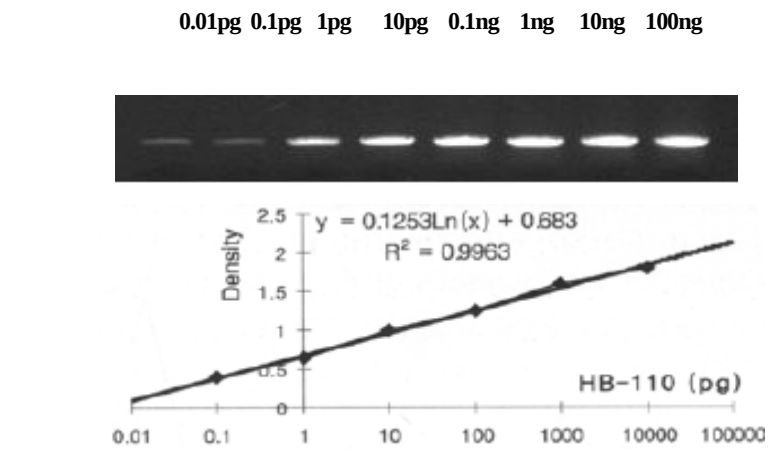


Fig.15. (a) Gel picture of PCR bands for serial dilution of standards and a calibration curve

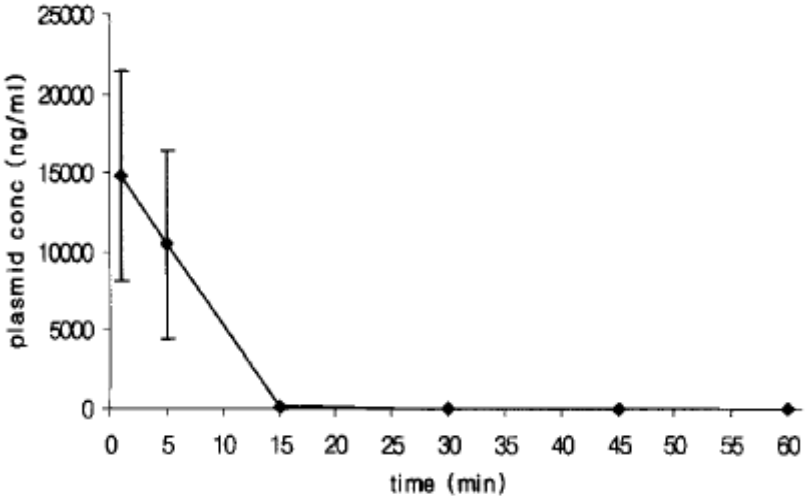


Fig.15. (b) Plasmid levels in the blood after the intravenous administration of 100 μl of HB-110 (100 ug of plasmid DNA) to mice. The data represents average levels ± SD from five mice at each time-point.

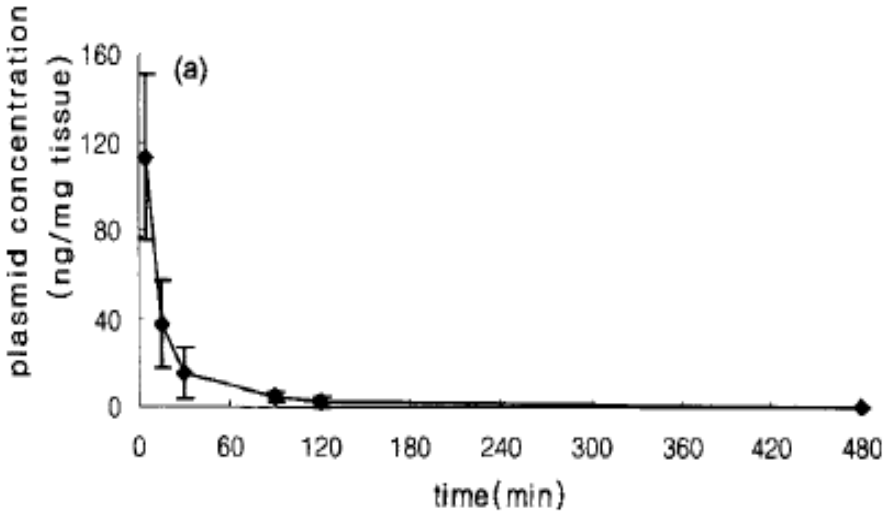


Fig.16. (a) Plasmid levels in the muscle after the intramuscular administration of 50 μl of HB-110 (100 ug of plasmid DNA) to mice. The data represents the average levels ±SD from five mice at each time-point.

Table 17. Tissue distribution of HB-110 after intramuscular administration

Tissue	90 m	8 h	1d	11 d	14 d	30 d
Liver	5/5	5/5	0/5	0/5	0/5	0/5
Brain	3/5	0/5	0/5	0/5	0/5	0/5
Kidney	4/5	0/5	0/5	0/5	0/5	0/5
spleen	4/5	4/5	0/5	0/5	0/5	0/5
Heart	3/5	2/5	0/5	0/5	0/5	0/5
Testis	3/5	3/5	0/5	0/5	0/5	0/5
Lung	4/5	4/5	0/5	0/5	0/5	0/5
muscle	5/5	5/5	2/5	1/5	0/5	0/5

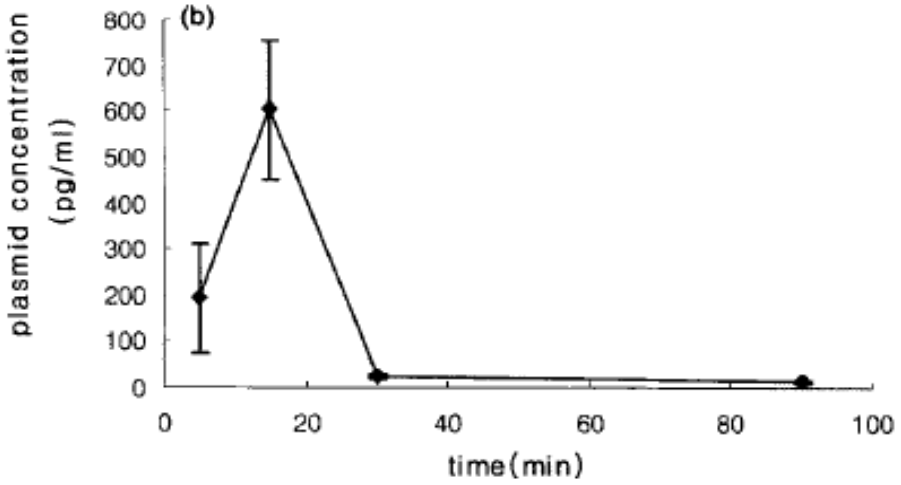


Fig.16.(b) Plasmid levels in the blood after the intramuscular administration of 50 μl of HB-110 (100 ug of plasmid DNA) to mice. The data represents the average levels ±SD from five mice at each time-point.

3.6.3 Tissue Distribution of HB-110 after Intramuscular Administration

Plasmid DNA at the injected muscle spreads to other tissue through the blood circulation. The HB-110 concentration was measured at several times in seven tissues, liver, lung, kidney, heart, spleen, testis and brain (Fig.17).

A previous study found that the plasmid concentration in tissue other than the administration site peaked at 90 min after administration. In this study, the highest concentration of plasmid DNA was detected in the liver at 90 min (24.172 pg/mg tissue). There were also considerable amounts in the lung (9.467 pg/mg tissue) and spleen (7.688 pg/mg tissue), while there was an infinitesimal level (<0.59 pg/ mg tissue) in the other organs (Table 18). The level of plasmid DNA in the tissues decreased rapidly and was undetectable 24 h after administration. Although a significant amount of plasmid DNA was detected in the liver compared with other organs, the amounts were extremely low compared with the quantity in the muscle (4570 pg/mg tissue) at 90 min post-injection.

3.6.4 Evaluation of Biodistribution using RT-PCR

In quantitative real-time PCR (qPCR) for the analysis of residual HB-110 in the tissues of rats, the limit of detection was approximately 10 plasmid DNA copies / 100 ng total DNA. The quantitation between enhanced fluorescence signal of the probe and concentration was identified at the interval between 10 copies and 10^7 copies. All biological sample analyses were accompanied by reference material tests so that the standard curve could be estimated and used for each.

3.6.4.1 Pharmacokinetics after a Single Dose

A single dose of CHB therapeutic DNA vaccine (HB-110) was administered intramuscularly in the tibialis anterior muscle of Sprague-Dawley (SD) rats, and *in vivo*

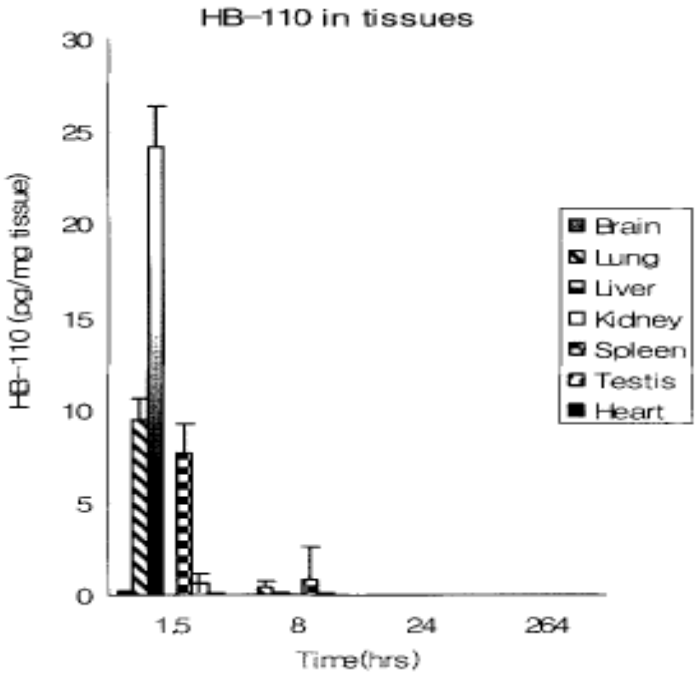


Fig.17. The tissue distribution of HB-110 after the intramuscular administration of 50 μl of HB-110 (100 μg of plasmid DNA) to mice. The data represents the average levels ±SD from five mice at each time-point.

Table 18. The amounts of HB-110 in the organs 90 min after intramuscular administration

Tissue	Concentration (pg/mg tissue)
Liver	24.172
Brain	0.072
Kidney	0.014
spleen	7.688
Heart	0.038
Testis	0.591
Lung	9.467
Muscle	4570

distribution of the test material was quantified using a real-time PCR assay whose limit of quantification (LOQ) was 100 copies/μg genomic DNA. After HB-110 was administered to 10 rats per group (5 male and female each) 100μg/head, general symptoms were observed and 11 tissues, including the injection site, were analyzed at day 1, 7, 30 and 60.

Residual plasmid DNA in the tissues was as follows:

- No abnormality was found among general symptoms.
- Residual plasmid DNA at the injection site appeared continuously decreasing, leaving less than 1,000 copies/μg genomic DNA from day 30.

As for the residual HB-110 in tissues other than the injection site, it was detected for up to 7 days after the administration in tissues other than the spleen and bone marrow. HB-110 in the spleen and bone marrow was detected up to 30 days but the level was less than 1,000 copies/μg genomic DNA.

Taken together, *in vivo* distribution of CHB therapeutic DNA vaccine (HB-110) was reduced with time, under the specified test conditions, when a single dose was intramuscularly administered in the tibialis anterior muscle of male and female SD rats. Plasmid DNA does not seem to be accumulated in the brain, testis, ovary, heart, kidney, lymph node, lungs, spleen, liver and bone marrow as well as the injection site (Fig.18).

3.6.4.2 Pharmacokinetics after a Repeated Dose

CHB therapeutic DNA vaccine (HB-110) was administered intramuscularly 3 times at 2 week intervals in the tibialis anterior muscle of Sprague-Dawley (SD) rats, and *in vivo* distribution of the test material was quantified using a real-time PCR assay whose limit of quantification (LOQ) was 100 copies/μg genomic DNA. After HB-110 was administered to 10 rats (5 male and female each) 100 μg/head, general symptoms were observed and 11 tissues, including the injection site, were analyzed at day 7, 30 and 60. Residual plasmid

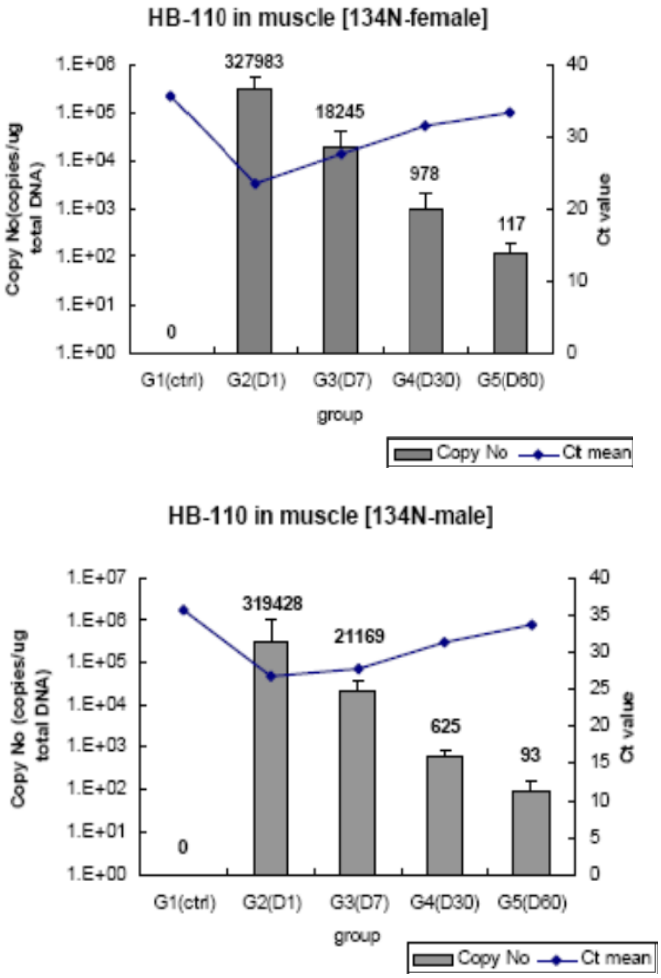


Fig.18. Pharmacokinetics after a single dose administration

DNA in the tissues was as follows:

- No abnormality was found among general symptoms.

- Residual plasmid DNA at the injection site appeared continuously decreasing until day 60, at which point 147 and 315 copies/μg genomic DNA were detected in male and female rats, respectively. Residual plasmid DNA was not detected on day 60 in tissues other than the injection site. Less than 1,000 copies/μg genomic DNA was detected on day 7 and 30.

Taken together, *in vivo* distribution of CHB therapeutic DNA vaccine (HB-110) was reduced with time, under the specified test conditions, when it was administered 3 times at 2 week intervals in the tibialis anterior muscle of male and female SD rats. Plasmid DNA does not seem to be accumulated in the brain, testis, ovary, heart, kidney, lymph node, lungs, spleen, liver and bone marrow as well as the injection site (Fig. 19).

When HB-110 was administered in rats 3 times at 2-week intervals, residual HB-110 plasmid copies were approximately ≤315 copies throughout 60 days, as confirmed by RT-PCR method, which can quantify plasmids of up to 10 copies/ug genomic DNA. This level is much lower than the standard recommended by the FDA and KFDA (30,000 copies/ug genomic DNA). Therefore, it was deemed possible that HB-110 could be exempted from genomic integration study.

3.7 HB-110 Manufacturing for Phase 1 Clinical Trial

Two lots of HB-110 clinical material were manufactured using the GMP facility in Dalseong Plant of Dong-A Pharm. for the clinical trial and were provided to the Catholic University of Korea Seoul St. Mary's Hospital.

Quality control tests were performed for the GMP production material in accordance with the established specifications and test procedures. According to the results, more than 95% was supercoiled monomer, and HCD was 1ng/mg DNA, indicating excellent purity

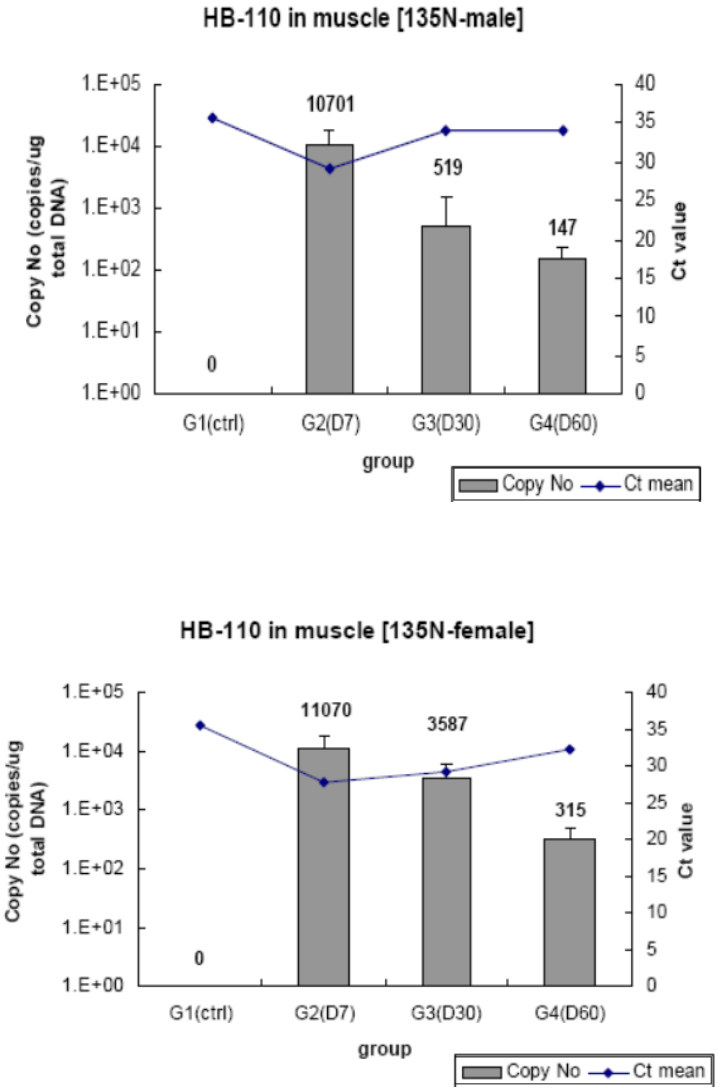


Fig.19. Pharmacokinetics after a repeated dose administration

and shape (Table 19).

As the HB-110 sample manufactured for phase 1 clinical study was found as appropriate in the QC results, it was deemed possible to use it in patients participating in phase 1 clinical study.

3.8 Phase 1 Clinical Trial

A phase 1 clinical study (Fig.20) using the HB-110 clinical sample with confirmed QC eligibility was performed in 27 patients with HBV infection, and HB-110 was not associated with significant side effects (Appendix 3).

Among 18 patients who used HB-110, 4 of them had e antigen seroconversion, which lasted until the end of the study(Appendix 4). In Adefovir group, 1 out of 9 subjects had e antigen seroconversion, which disappeared in a month.

This phase 1 clinical study of HB-110 confirmed the safety and therapeutic possibility of HB-110 in patients with HBV infection and provided useful data for future phase 2 clinical study.

3.9 Study of Electroporation Application

3.9.1 HBs Antigen Expression

TA muscles were collected on days 2, 5, 10, and 30 after vaccination with a single dose. A control mouse group was administered DNA only, without electroporation (referred to as the direct injection group, DI group). In both groups, the peak levels of HBs Ag were achieved on day 10. However, the electroporation (EP) group evidenced a more rapid increase of antigen expression; the antigen level at day 5 was equivalent to that of day 10. The increase in expression levels achieved by electroporation was substantial, with the

Table 19. Quality control test results of the clinical materials

Item		Lot No. VB70901	Lot No. VB80702
Appearance		Acceptable	Acceptable
pH		7.0	7.0
Restriction enzyme DNA fragment		Acceptable	Acceptable
Purity	Spectroscopy	1.93	1.86
	Molecular conformation	98.7%	98.0%
	Host cell-derived peptide	0.155 ng/mg DNA	1.06 ng/mg DNA
	Host cell-derived DNA	0.0025 mg / mg DNA	0.004 mg / mg DNA
	Host cell-derived RNA	≤0.001 mg / mg DNA	≤0.001 mg / mg DNA
Titer	HBsAg expression	2,064 pg/mg DNA	2,203 pg/mg DNA
	HBcAg expression	3,158 pg/mg DNA	4,809 pg/mg DNA
	IL-12 expression	158.04 pg/mg DNA	160.13 pg/mg DNA
	mRNA expression	Acceptable	Acceptable
	Western blot	Acceptable	Acceptable
Sterility		Acceptable	Acceptable
Bacterial endotoxin		0.74 EU/mg DNA	0.85 EU/mg DNA
Abnormal toxicity		Acceptable	Acceptable
Pyrogen		Acceptable	Acceptable
Content		2.13 mg/ml	1.97 mg/ml
Insoluble foreign matter		Acceptable	Acceptable
Volume of injection in container		Acceptable	Acceptable

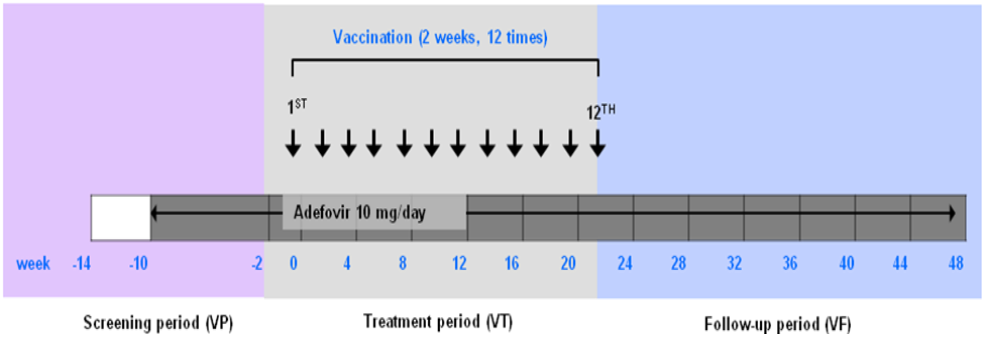


Fig.20. Phase 1 clinical trial protocol of HB-110. Eligible subjects were first treated with Adefovir [41]10mg/d for 8 weeks, and then the patients whose viral load decreased by 10 times were randomized to one of 4 groups. A total of 18 subjects received HB-110 2mg (n=6), 4mg (n=6) or 8mg (n=6) by intramuscular injection alternating between D and GM every 2 weeks (total 12 doses), along with 10mg of Adefovir (Fig.9.). Nine subjects in the control group received 10mg/d of Aadefovir alone.

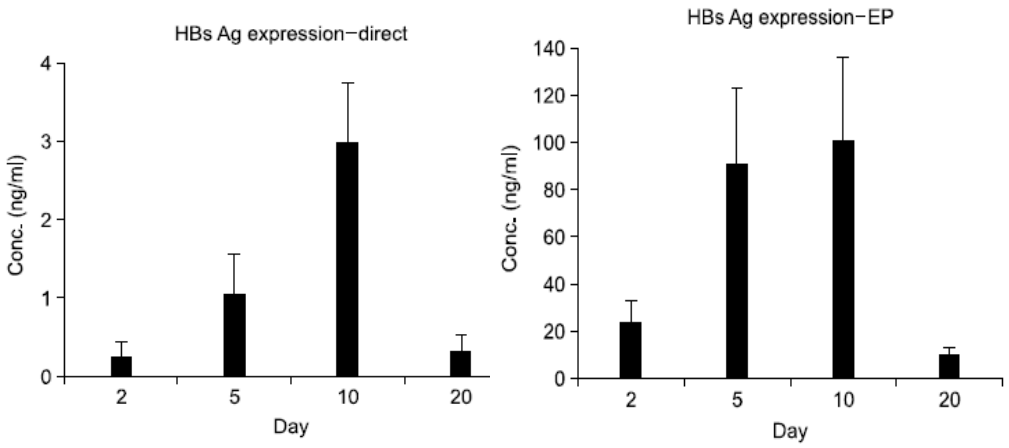
HBsAg levels in the EP group being 30 to 90-fold higher (Fig.21).

3.9.2 Antibody Responses

The effect of electroporation on antibody response was assessed by measuring the blood level of anti-HBs and anti-HBc on weeks 0, 3, 4, 6, 12. Immunizations were given on weeks 0 and 4. Humoral immunity was investigated over a time course and with dose escalations (Fig.22, Fig.23). Anti-HBs antibody response was detectable after the 2nd injection. The EP group evidenced a 3 to 8-fold enhancement in humoral response as compared to the DI (direct injection) group, and the magnitude of the response increased until week 12 (Fig.22(A)). Anti-HBc antibody response was detectable after the initial injection and was boosted further after the 2nd injection. Peak Ab levels were reached at 6 weeks and were sustained until 12 weeks in both groups (Fig.23(A)). The effect of electroporation on antibody response was less pronounced for HBc, when compared with the results of the anti-HBs. However, the antibody responses were still consistently higher in the EP group.

In the dose escalation study, four dosages (10, 20, 50, 100 ug of DNA) were evaluated. The titer of anti-HBs antibody in the EP group was increased up to the higher limit of the investigated range, but the DI group evidenced substantially lower responses for all dosages (Fig.22(B)). However, dose responses of anti-HBc antibody were observed in both the EP and DI groups at the lower ranges of the investigated dosages. The anti-HBc antibody response was saturated at 10 ug/head in the EP group and at 20 ug/head in the DI group (Fig.23(B)).

(A) (B)



C)

	Direct (ng/ml)	*EP (ng/ml)*	**EP/Direct**
DAY 2	0.2505	23.4388	**93.55**
DAY 5	1.0608	90.5755	**85.38**
DAY 10	2.9691	99.7986	**33.61**
DAY 20	0.3144	10.2302	**32.54**

Fig.21. HBs antigen expression. HBsAg expression in TA muscles on days 2, 5, 10, and 30 after vaccination with a single dose. DI group (A) and EP group (B) achieved the peak levels of HBs Ag on day 10. However, the EP group showed a more rapid increase of antigen expression; the antigen level at day 5 was equivalent to that of day 10. The HBsAg levels in the EP group being 30 to 90-fold higher than those of DI group (C).

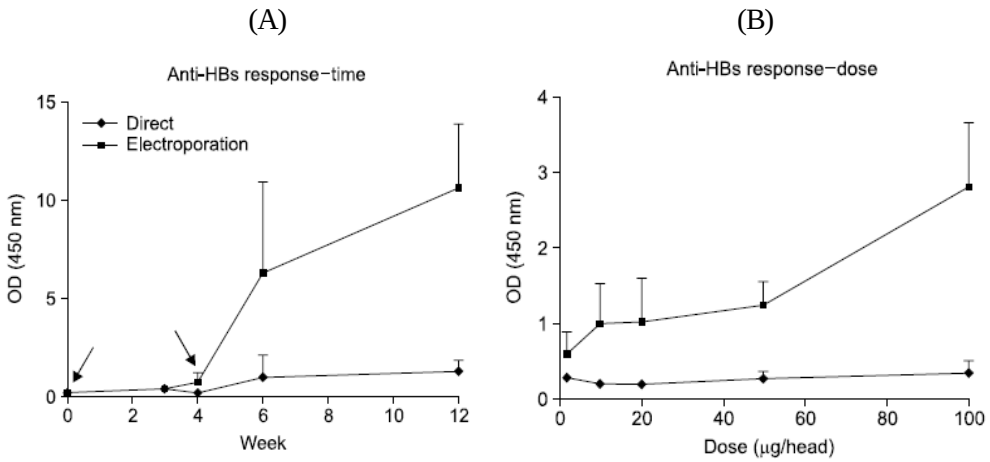


Fig.22. Anti-HBs responses. Anti-HBs responses. Mice vaccinated twice at 4-week intervals (depicted as arrows) were sacrificed on weeks 3, 4, 6, and 12. Five mice were used per group. The OD value was directly proportional to the quantity of anti-HBs antibodies. Anti-HBs antibody response was detectable after the 2nd injection. The EP group showed a 3 to 8-fold enhancement in humoral response as compared to the DI group, and the magnitude of the response increased until week 12 (A). In the dose escalation study, four dosages (10, 20, 50, 100 ug of DNA) were evaluated on week 12. The titer of anti-HBs antibody in the EP group was increased up to the higher limit of the investigated range, but the DI group evidenced substantially lower responses for all dosages (B).

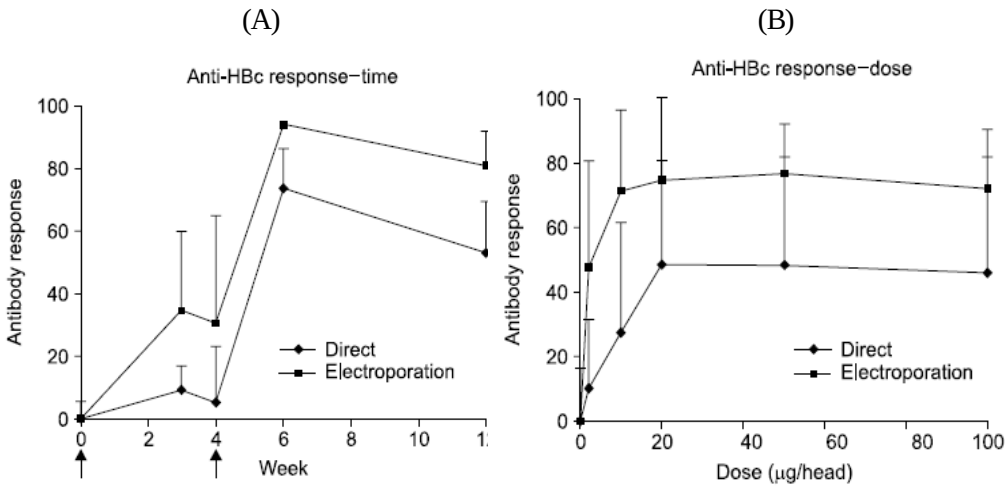


Fig.23. Anti-HBc responses. Mice vaccinated twice at 4-week intervals (depicted as arrows) were sacrificed on weeks 3, 4, 6, and 12. Five mice were used per group. Since the assay used to measure antibody response is a competing ELISA, antibody response is defined as $100x(1-OD_{sample}/OD_{normal\ plasma})$, which is proportional to the amount of anti-HBc antibodies. Anti-HBc antibody response was detectable after the initial injection and was boosted further after the 2nd injection. Peak Ab levels were reached at 6 weeks and were sustained until 12 weeks in both groups (A). Dose responses of anti-HBc antibody were observed in both the EP and DI groups at the lower ranges of the investigated dosages. The anti-HBc antibody response was saturated at 10 ug/head in the EP group and at 20 ug/head in the DI group (B).

3. 9. 3 Cellular Immune Response

Cellular immunity is the most important aspect with regard to the determination of the efficacy of a therapeutic DNA vaccine. Thus, cellular immune response was evaluated under several experimental conditions with different numbers and intervals of DNA vaccination. Here, the results of two protocols, three vaccinations at 2-week intervals (protocol 1) and two vaccinations at 4-week intervals (protocol 2), were compared. Cellular immune response was evaluated by counting the number of antigen-specific IFN-r secreting cells. The cellular immunity of HB-110 was enhanced after electric pulse by 1.78-fold for the S antigen and 3-fold for other antigens (preS, core, pol) in protocol 1 (3 times/2 wk-interval) (Fig.24(A)). However, the increase in magnitude due to electroporation was far larger in protocol 2 (twice/4 wk-intervals) than in protocol 1. In the second protocol, electroporation augmented the immune response by 3-fold, 13-fold, 7-fold, and 11-fold for S, preS, Core, and Pol, respectively (Fig.24(B)). The differing potency of electroporation for cellular immunity in the two protocols can be explained as follows.

In the DI groups we noted a marked difference between protocol 1 and protocol 2, the former inducing a stronger immune response, presumably as the consequence of the increased immunization frequency (Fig.25(A)). In the EP groups, the absolute immune response levels were similar for both protocols, thereby indicating that a high level of immune response can be achieved without frequent immunizations (Fig.25(B)).

Furthermore, electroporation evidenced a broader repertoire of cellular immune responses against all of the antigens, including subdominant antigens such as preS, Core, and Pol, although the magnitude was not as large as with the S antigen.

Electroporation elicited an overall 2-fold increase in cellular immunity in the EP group of protocol 2 (administered two vaccinations at 4-week intervals) as compared to the DI group of protocol 1 (administered three vaccinations at 2-week intervals) (Fig.25(C)).

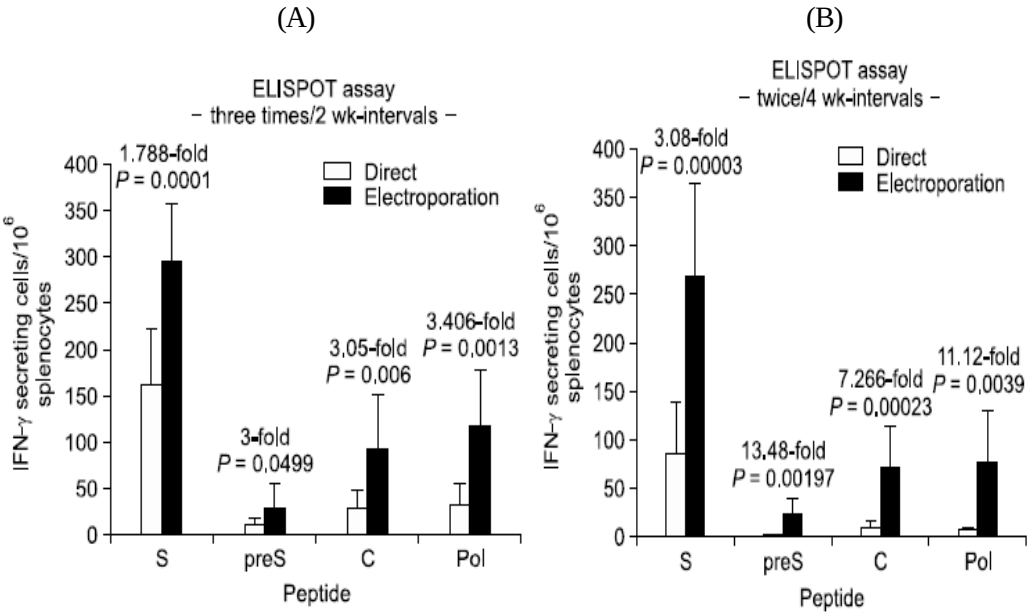


Fig.24. Cellular immune responses in protocol 1(A) and protocol 2(B). Cellular immune response was evaluated by counting the number of antigen-specific IFN-r secreting cells. The cellular immunity of HB-110 was enhanced after electric pulse by 1.78-fold for the S antigen and 3-fold for other antigens (preS, core, pol) in protocol 1 (3 times/2 wk-interval) (A). In the second protocol (twice/4 wk-interval), electroporation augmented the immune response by 3-fold, 13-fold, 7-fold, and 11-fold for S, preS, Core, and Pol, respectively (B).

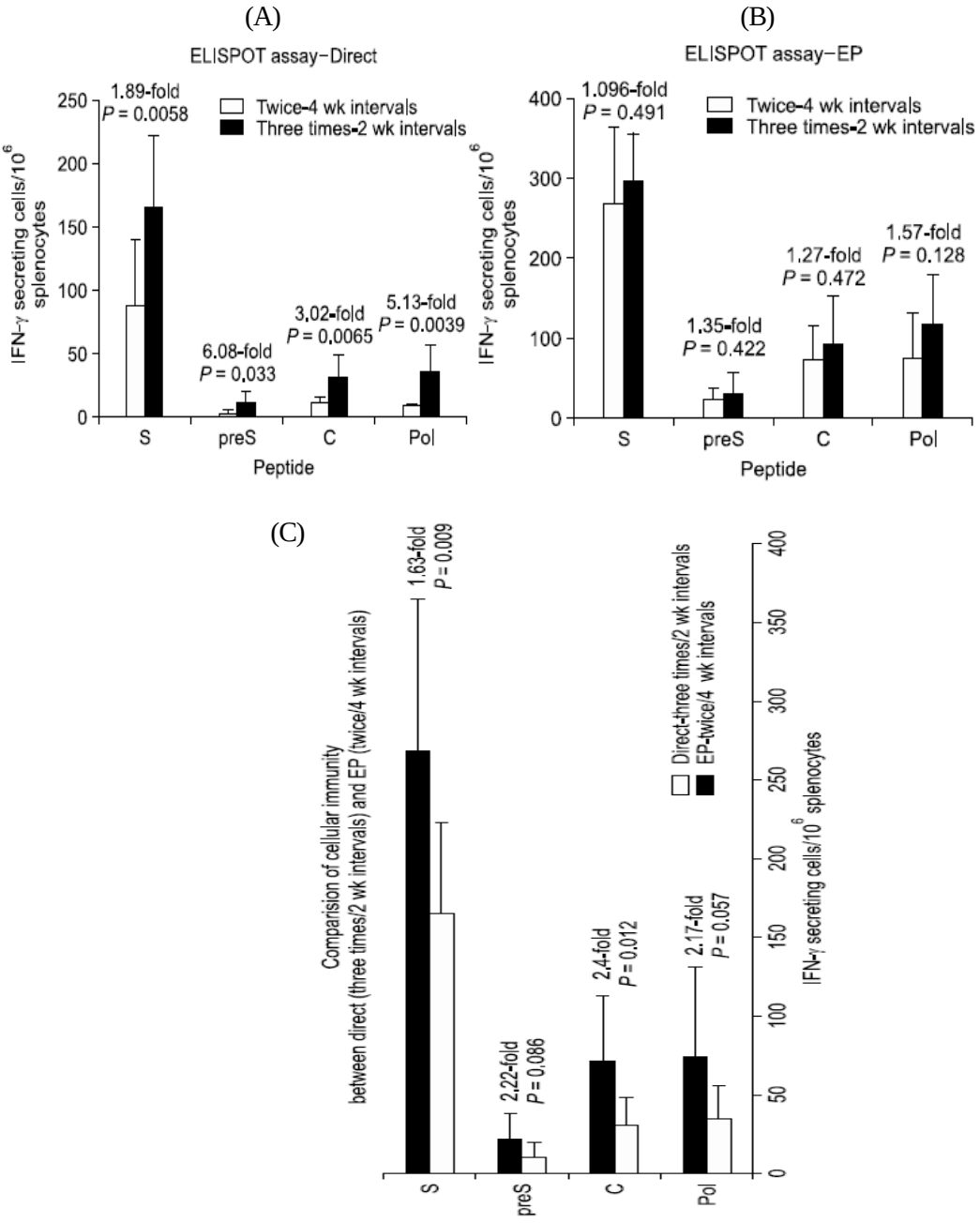


Fig.25. The differing potency of electroporation for cellular immunity in the two protocols and the overall effect on cellular immunity by electroporation. In the DI groups we noted a marked difference between protocol 1 and protocol 2, the former inducing a stronger

immune response, presumably as the consequence of the increased immunization frequency (A). In the EP groups, the absolute immune response levels were similar for both protocols, thereby indicating that a high level of immune response can be achieved without frequent immunizations (B). Electroporation elicited an overall 2-fold increase in cellular immunity in the EP group of protocol 2 as compared to the DI group of protocol 1 C).

3. 9. 4 HBsAg Seroconversion Analysis

Since the appearance of anti-HBs antibodies is a serological marker associated with recovery from natural HBV infection, we investigated whether the humoral response elicited by EP (Fig.22) would be of immunological relevance in a transgenic mouse model of chronic hepatitis B. After three vaccinations of transgenic mice, 50% of mice receiving vaccine in combination with EP showed HBsAg seroconversion as defined by the absence of HBsAg and the presence of anti-HBs antibody. In contrast, only one case of seroconversion was observed in the group receiving vaccination alone (Fig.26). The anti-HBs antibody titer in the vaccine-plus-electroporation group was about 1.7-fold higher than in the vaccination-alone group (data not shown).

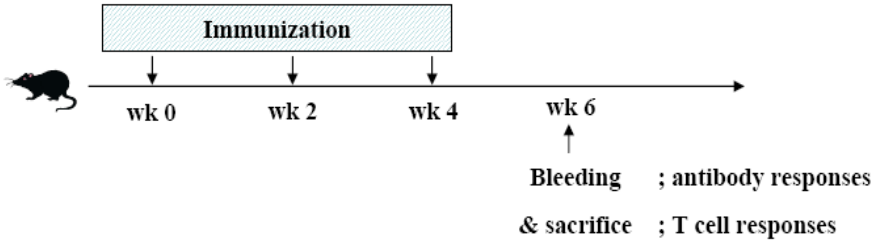
3. 9. 5 Conclusion of Electroporation Study

In the electroporation study of HB-110 in a mouse model, HB-110 was found to increase the expression of antigen, antibody production and cellular immune response markedly compared with the direct administration group. S antigen seroconversion rate was higher in the electroporation group in the HBV transgenic mouse model as well.

Electroporation method is expected to enhance the efficacy of HB-110 in phase 2 clinical study as well, and the dose and interval of HB-110 administration may be reduced for better convenience and lower cost of treatment.

(A)

Gr	#	The scheme of therapy	Route	Etc.
1	M2 / F2	pGX10 (50μg)	IM	
2	M3 / F3	HB-110 (50μg)	IM	
3	M3 / F3	HB-110 (50μg)	IM → elec	



(B)

Group	HBsAg seroconversion† rate
pGX10	**0/6**
HB-110 (DI)	**1/6**
HB-110 (EP)	**3/6**

† HBsAg seroconversion means the disappearance of serum HBsAg and the induction of anti-HBs.

Fig.26. The HBsAg seroconversion rate was investigated in transgenic mice that constitutively express HBsAg in the liver. Mice were divided into three groups and given either pGX10 (vector control), HB-110 or HB-110 with electroporation. Mice were vaccinated three times at 2-week intervals and blood was collected at 2 weeks after final vaccination (A). Plasma was isolated and analyzed for HBsAg and anti-HBs antibody. In the vaccine-plus-electroporation group, three mice (50%) showed HBsAg seroconversion. Only one case of seroconversion was observed in the group receiving only vaccine (B).

IV. DISCUSSION

Most of the existing HBV prophylactic vaccines use protein antigens to induce a primary humoral immune response, which can be rapidly re-called upon HBV entry to eliminate the virus before establishing an acute or chronic infection. Since the HBV therapeutic vaccine developed in this project aimed at eliminating or inhibiting the proliferation of HBV that has already established a chronic infection in the body, its general mechanism of action had to be designed differently from the existing prophylactic vaccines.

The most important aspect in a therapeutic vaccine for eliminating a virus that is already proliferating in human body, such as HBV, is the induction of a specific cellular immune response to that virus. In other words, a therapeutic HBV vaccine should be designed to induce an effective cellular immune response. Here, various aspects of vaccine development including vaccine type, use of cytokine, gene engineering and methods for production and optimal administration were examined with the goal of enhancing the cellular immune response to HBV.

Reports so far have found that cellular immune response is induced by antigen presentation to CD8+ T cells by MHC I molecules of the cells expressing the antigen gene (Fig. 27). For the development of an HBV therapeutic vaccine, it is essential to select a vector that can effectively and safely induce a cellular immune response. After considering various types of vaccines, a naked plasmid vector (pGX10) was selected as the most suitable vector that would satisfy the requirements of safety, efficacy and manufacturability.

Different cytokines were evaluated for their ability to enhance cellular immunity, and IL-12 was selected as the most appropriate for enhancing cellular immunity induced by the therapeutic DNA vaccine (HB-110). According to studies conducted at POSTECH [34], a joint research institution of this study, p40, a subunit of IL-12, inhibits IL-12 activity when

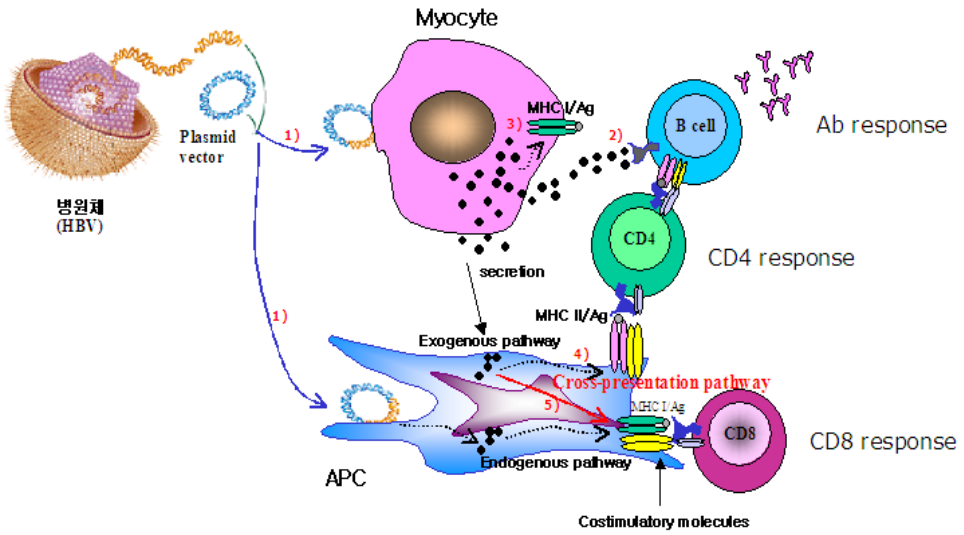


Fig.27. Inducing immune response by therapeutic DNA vaccine

it is singularly secreted to the outside of the cell. Thus Asn 222, a glycosylation site of p40 subunit, was converted to Leu to inhibit the release of p40 alone and increase IL-12 activity. When this mutant form of IL-12 (hIL-12m) was added to the HBV DNA vaccine in a mouse model, cellular immunity (as measured by ELIspot response) increased by about 1.5-3 times depending on the antigen.

To increase intracellular expression of HBV genes, codon optimization was performed for the HBV antigen genes. After the codon optimization, HBV gene expression rate was observed to increase approximately 1.5- to 3-fold.

E. coli is most widely used as a host for the production of naked plasmid DNAs in therapeutic DNA vaccines. Among various conformational isomers of plasmid DNA, supercoiled monomer is the most desirable form for intracellular delivery of plasmid DNA vaccine in human. To increase the formation of supercoiled monomer in the production process, optimization studies of the fermentation and purification steps were conducted. In the fermentation process study, supercoiled monomer formation could be increased by adding glycerol in the mid-log phase of the cell culture. In the purification process, supercoiled monomer content could be increased from 58% to 91% by adopting a thiophilic/aromatic adsorption (TAA) chromatography step.

Intracellular delivery is one of the fundamental bottlenecks in the application of naked plasmids for DNA vaccines. Thus, electroporation studies are currently ongoing to increase the intracellular delivery rate of plasmid DNA after intramuscular administration. In the studies conducted with HB-110, we found that electroporation could increase the expression of s antigen by about 30-90 times in a mouse model. Based on these encouraging results, a phase 2 clinical study that incorporates the electroporation method into the administration protocol is scheduled for the future.

In conclusion, a novel HBV therapeutic DNA vaccine was developed using naked

plasmid DNA vectors and including hIL-12 for enhanced cellular immunity. Codon optimization was applied to increase antigen expression, and the proportion of supercoiled monomer, which is appropriate for therapeutic DNA vaccine, was improved by optimizing the production process. Electroporation was investigated as a method to enhance intracellular delivery of the DNA vaccine, and was confirmed to be highly effective in increasing the cellular immune response after intramuscular administration. The electroporation method will be incorporated in a phase 2 clinical study and is expected to increase the clinical efficacy of the vaccine.

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국문 초록

현재까지 개발되어 사용되고 있는 Hepatitis B virus (HBV) 치료제들은 면역치료제인 Interferon-α 및 화학요법제인 Lamivudine, Adefovir, Entecarvir 등이 있다. Interferon-α 경우 투여시 부작용이 심하며, 화학요법제들은 바이러스 증식억제의 장점을 가지고 있으나, 환자에 장기간 투여시 독성, 내성바이러스 출현이 발생되며, 투여 중단시 HBV 의 recurrence 가 발생되는 제한이 있다. 본 연구에서는 현재 사용하는 치료제보다 유효성 및 안전성이 우수한 HBV 치료제를 개발하기 위해 HBV 감염자 대상으로 치료 DNA 백신 개발을 진행하고 있으며, 먼저 선도 물질인 HB-100 을 개발하여 우크라이나 및 리투아니아에서 사전임상을 실시하여 안전성 및 유효성을 확인하였다. 그리고 HB-100 보다 효능이 우수하며 경제적으로 생산이 가능한 HB-110 을 개발하였다.

본 연구에서는 HB-110 의 생산공정을 확립하였으며, 물리화학 및 생물학적 특성분석과 안정성, 독성, 역동력학 연구를 수행하여 전임상 연구를 완료하였다. HB-110 은 HBV 항원유전자 및 변이체 IL-12 유전자를 발현하는 3 종의 naked plasmid 로 이루어진 DNA 백신으로, *E. coli* DH5α에 각각의 plasmid 를 형질전환시켜 생산세포주 bank 를 제조하였다. 제조된 세포주 bank 를 대상으로 세포주의 유전형, 표현형 및 오염여부 등에 대해 시험을 수행하였으며, 적합하게 제조된 것을 확인하였다. 제조된 세포주를 이용하여 생산공정을 확립하였으며, 배양공정에서 배양중 glycerol 첨가와 정제공정단계에 thiophilic/aromatic adsorption 크로마토그래피를 실시하여 supercoiled monomer 형태가 90% 이상 순도의 plasmid 시료를 확보 할 수 있었다. 확립된 생산공정을 이용하여 생산된 HB-110 에 대해 유전자 치료제의 가이드를 적용하여 물리화학 및 생물학적 특성분석을 진행하였으며,

이를 기반으로 생산된 HB-110 이 의약품으로 사용 적합성을 판단하는 품질기준 시험법을 수립하였다. 독성시험은 확립된 생산공정으로 생산한 HB-110 을 대상으로 안전성 평가연구원 및 Ina(일본)에서 설치류 및 원숭이에서 평가를 실시하여, HB-110 이 안전한 물질임을 (NOAEL ≥ 4mg/kg) 확인하였다. 약동력학 연구는 마우스에서 실시하였으며, 정맥으로 투여 후 평가결과 half-life 가 1.9 분이며 AUC 가 103 ug min/ml 로 나타났다. 투여 경로인 근육내 투여시 근육내에서 약 11 일까지 HB-110 이 잔류되는 개체가 존재하였으며, 타장기에서는 투여 후 8 시간 이후에는 검출한계(0.01pg/mg tissue) 미만으로 나타났다. 이렇게 확보된 전임상 자료를 이용하여 제 1 상임상 시험계획서(IND)를 KFDA 에 제출하여 승인을 득하였으며, 카톡릭의대 강남성모병원에서 HBV 감염자 대상으로 1 상임상을 완료하였다.

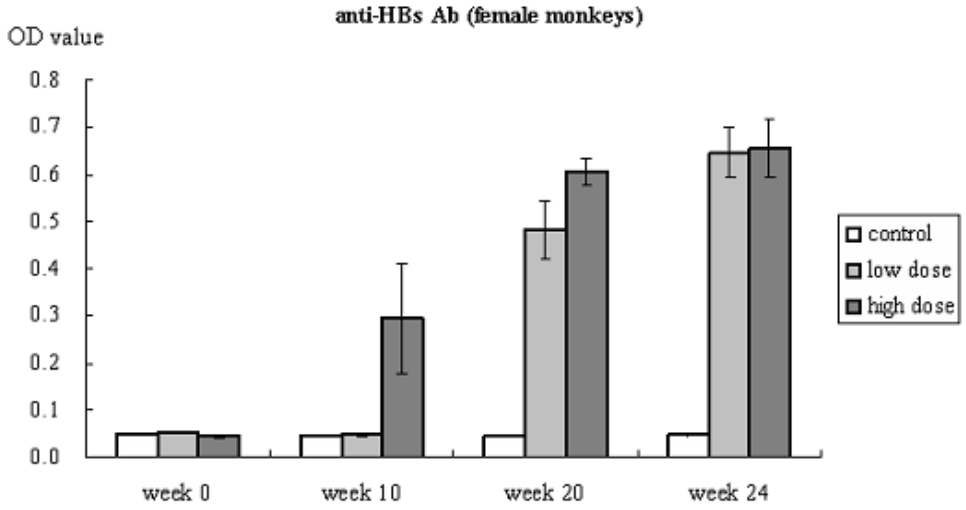
또한 본 연구에서는 HB-110 의 효능을 향상시키기 위해 전기천공법(electroporation) 적용연구를 실시하여, S 항원 유전자의 발현을 대폭적으로 증가시켰으며, 세포성 면역반응이 항원에 따라 약 1.7~3 배 이상 향상되는 것을 mouse 시험을 통해 확인하였다. 향후 2 상 임상시험에서는 전기천공법을 적용할 계획이며, HB-110 의 효능증가를 기대 할 수 있을 것으로 예측된다.

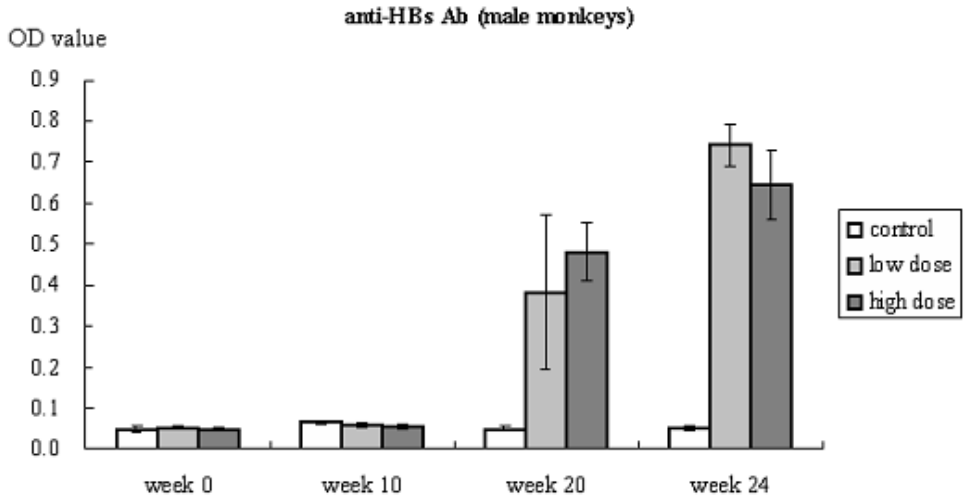
APPENDIX

Appendix 1. Safety assessment in small animals

Item	Content	Result
Single dose toxicity	• Toxicity after IM and IV administration in SD rats (maximum 4 mg/kg)	No toxicity
Repeated dose toxicity	• Toxicity after 26-week repeated administration in SD rats (maximum 4 mg/kg)	No toxicity
Genetic toxicity	• PCR assay to determine whether plasmid DNA was inserted in the host chromosomal DNA of the injection site (IM) cells	Negative
Reproductive toxicity	• Reproductive gene expression identified by RT-PCR	Negative
Autoimmune toxicity	• Antibody level (ELISA) and the number of antibody forming cells (ELISpot) for plasmid DNA itself	No toxicity

Appendix 2. Identification of anti-HBs Ab after 6 month repeated HB-110 dose in monkeys





Appendix 3. Safety results of HB-110 phase 1 clinical trial

Group	Adverse Event	Adverse Drug Reaction (related to HB-110)	Drop-out
Adefovir	5 (2 patients)	NA	2/9*
Adefovir + HB-110 2 mg	2 (1 patients)	0	0/6
Adefovir + HB-110 4 mg	4 (3 patients)	0	0/6
Adefovir + HB-110 8 mg	9 (2 patients)	0	0/6

* Two cases of drop-out in the Adefovir monotherapy group (n=9)

Appendix 4. HBe Ag seroconversion rate of HB-110 phase 1 clinical trial

Intervention group	HBeAg seroconversion (Follow-up at 26 weeks after the administration was discontinued)
Adefovir monotherapy	0/9 (0%)
Adefovir + DNA vaccine 2 mg	2/6 (33%)
Adefovir + DNA vaccine 4 mg	1/6 (17%)
Adefovir + DNA vaccine 8 mg	1/6 (17%)
Adefovir + DNA vaccine (all groups combined)	4/18 (22%)

Appendix 5. Publications

1. **Chae Young Kim**, Eun Sung Kang, Seon Beom Kim, Han Eol Kim, Jae Hoon Choi, Dong Sop Lee, Se Jin Im, Se Hwan Yang, Young Chul Sung, Byong Moon Kim and Byung-Gee Kim, Increased in vivo immunological potency of HB-110, a novel therapeutic HBV DNA vaccine, by electroporation. Experimental and Molecular Medicine 40(6):669-676 (2008)
2. Eun Sung Kang, **Chae Young Kim**, Seon Beom Kim, Se Jin Im, Se Hwan Yang, Young Chul Sung, and Byong Moon Kim, In vivo Kinetics and Biodistribution of HB-110, a Novel HBV DNA vaccine, after Administration in Mice. Arch Pharm Res 30(3):355-360 (2007)
3. S-H Yang, C-G Lee, S-H Park, S-J Im, Y-M Kim, J-M Son, J-S Wang, S-K Yoon, M-K Song, A Ambrozaitis, N Kharchenko, Y-D Yun, C-M Kim, **C-Y Kim,** S-H Lee, B-M Kim, W-B Kim and Y-C Sung, Corration of antiviral T-cell responses with suppression of viral rebound in chronic hepatitis B carriers : a proof-of concept study. Gene Theerapy 13: 1110-1117 (2006)
4. Seung Kew Yoon, Yong Bok Seo, Se Jin Im, si Hyun Bae, Myeong Jun Song, Chan Ran You, Jung Won Jang, Se Hwan Yang, You Suk Suh, Ji Soo Song, Byong Moon Kim, **Chae Young Kim**, Sook Hyang Jeong and Young Chul Sung, Safety and immunogenicity of therapeutic DNA vaccine with antiviral drug in chronic HBV patients and its immunogenicity in mice. Liver International (2014): 1-11