Therapeutic efficacy of hepatitis B surface antigen–antibodies-recombinant DNA composite in HBsAg transgenic mice

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Abstract

Therapeutic efficacy of HBsAg-anti-HBs-recombinant DNA harboring hepatitis B virus (HBV) S gene complex was compared with three other therapeutic vaccine candidates (recombinant HBsAg, HBsAg complexed to anti-HBs antibodies and naked plasmid DNA encoding the HBV S gene). After four injections at 3-week intervals, the most pronounced decrease of serum HBsAg, the highest titer of anti-HBs response, the highest level of interferon-γ produced by splenocytes and potent cytotoxicity T cell response were observed in the HBsAg-anti-HBs-sDNA immunized group. Reduced expression of HBsAg in hepatocytes was also shown. The therapeutic mechanism of HBsAg-anti-HBs-DNA was speculated as modulation of HBsAg presentation via both endogenous and exogenous pathways. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Hepatitis B virus; Therapeutic vaccine; Immune tolerance

1. Introduction

Hepatitis B virus (HBV)-related hepatitis is a necroinflammatory liver disease of variable severity. In HBV-endemic areas, persistent HBV infection either associated with a healthy carrier state or with chronic liver diseases remains to be a serious risk for development of liver cirrhosis and hepatocellular carcinoma. Several therapeutic vaccine candidates have been developed and some are presently under clinical trial [1–6]. A number of laboratories have developed different lineages of HBV-transgenic mice and these have been used for immunological studies. However, because these mice express HBV from different constructs of the full-length or selected parts of the HBV genome, direct comparisons between data of the various laboratories is difficult to be made. Due to the similarity of transgenic mice to human HBV-carriage and the well-defined immunological background in mice, transgenic mice is still a widely used model for evaluation of immunotherapy [7]. In this study, we used the C57BL/6J-TgN transgenic mice which harbor the gene encoding S, PreS1 and PreS2 domains of HBV, and express HBsAg in serum, liver and kidney tissues to mimic a human chronic HBsAg carrier state, but with no virus replication. To compare the therapeutic efficacy of different vaccine candidates in this lineage of transgenic mice, we used four types of immunogens, namely: recombinant HBsAg, HBsAg-anti-HBs immunogenic complex (IC), naked plasmid DNA harboring the S gene of HBV (s-DNA), and IC in combination with s-DNA (IC–sDNA). Results showed that IC–sDNA immunization was most effective in decreasing serum HBsAg, eliciting anti-HBs, inducing Th1-type cell-mediated immune response, and down-regulating the expression of HBsAg in hepatocytes.
2. Materials and methods

2.1. Mice

C57BL/6J-TgN (Alb1HBV) 44 Bri mice (H-2b), checked for serum HBsAg positive, anti-HBs negative, and HBsAg positive in the liver and kidney tissues (after being sacrificed), were provided by The Jackson Laboratory (USA). A total of 28 transgenic mice (13 males, and 15 females), 8–12 weeks of age, weight, 16–18 g were used in this study. Normal C57BL/6J mice (H-2b) were bred under standard pathogen-free conditions in the Laboratory Animal Unit of the University of Hong Kong. All mice were housed in cages under standard conditions. The criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication 86-23, 1985) were followed.

2.2. Immunogens

Recombinant yeast-derived HBsAg (lot YHB 9811223): commercial yeast-derived recombinant hepatitis B vaccine was provided by Beijing Institute of Biological Products (China).

HBsAg-mouse anti-HBs IC: the source of HBsAg used for preparation of IC was from the same lot of vaccine as stated above. The mouse anti-HBs antibodies used were provided by our own laboratory. IC was prepared in excess of HBsAg, as described by Qu et al. [8].

Recombinant plasmid DNA with insertion of HBV S gene driven by cytomegalovirus immediate early promoter (s-DNA) was a generous gift from Whalen [9]. Plasmid DNA was amplified and purified by anion exchange column (Qiagen, Hilden, Germany), and finally, resuspended in endotoxin-free sterile physiological saline for injection. All plasmid DNA used were checked for endotoxin (less than 0.25 endotoxin unit/p109 g) prior to immunization. IC–sDNA was prepared by combining naked plasmid DNA with IC at appropriate ratio.

2.3. Immunization

Twenty-eight HBsAg transgenic mice were numbered and randomly divided into five groups and immunized with different immunogens (Table 1). To exclude the effect of anesthesia over the immune response in mice, all immunized mice were anesthetized with identical dose of sodium barbital, and all immunogens were injected into the tibialis anterior muscle of both hind legs of mice. The immunization was given in four doses every 3 weeks over 12 weeks, and on week 14, mice were boosted with the same immunogen 7 days prior to sacrificing the mice for cell-mediated immune response assay.

2.4. Determination of immune responses

Serum samples were taken before each dose of immunization for the determination of HBsAg and anti-HBs. Both serum HBsAg and anti-HBs were assayed by ELISA (BIOKIT, S.A. Spain). For HBsAg quantification a panel of HBsAg calibrators (Abbott Diagnostics, Chicago) was applied in the assay. The level of anti-HBs was quantified using standard positive controls (10–100 mIU/ml) provided with the kits. The animals were sacrificed on week 15. The spleen cells from all animals were assayed for HBsAg specific Th1 and Th2 cell cytokines. 5 × 10^5 splenocytes from each mouse were cultured in 10% calf serum-RPMI 1640, stimulated with 10 µg/ml of recombinant HBsAg at 37°C for 3 days, and supernatants of cultured cells were collected and interferon-γ and interleukin-4 were assayed by ELISA using OptEIA kits (PharMingen, USA).

Cells were further cultured by adding 25 IU/ml of murine recombinant IL-2 (R&D Systems, USA) for additional 4–5 days to expand specific T cells. The cytotoxicity T cell (CTL) activity of the splenocytes was measured in triplicates using a standard 4 h calcein release assay in U-bottom 96-well microplates [10–12]. Target cells used in CTL assays were the splenocytes of

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Immunogens</th>
<th>Dose (per mouse)</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>1</td>
<td>HBsAg + alum</td>
<td>2 µg HBsAg</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>IC + alum</td>
<td>2 µg HBsAg</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>IC–sDNA</td>
<td>2 µg HBsAg + 100 µg sDNA</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>s-DNA</td>
<td>100 µg sDNA</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Unimmunized</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

a IC – HBsAg-anti-HBs complex.
b sDNA – recombinant plasmid DNA harboring S gene.
c NA – non-applicable.
normal C57/6J, infected either with 10 PFU/cell of a recombinant vaccinia virus which harbored the HBsAg gene (vaccinia–HBsAg virus, abbreviated as Vac–HBsAg) or with vaccinia virus (Vac, negative control) for 12 h. Target cells were labeled immediately before use by incubating cells in 2 μM calcein AM (molecular Probes Inc., USA) for 40 min at 37°C. The expanded effector spleen cells were purified and resuspended in 10% calf serum-RPMI 1640, mixed with 5000 calcein AM labeled targets, at effector/target (E:T) ratios of 100/0.3. The plates were centrifuged at 100 × g for 3 min and further incubated at 37°C for 4 h. The cytolysis of the targets was determined by measuring the fluorescence intensity (FI). The percentages of specific cytolysis were calculated as follows:

\[
\left(1 - \frac{\text{Experimental FI} - \text{Total lysis FI}}{\text{Target control FI} - \text{Total lysis FI}}\right) \times 100\%.
\]

2.5. Immunohistopathological study

After sacrificing the mice, liver and kidney tissues were either snap frozen in liquid nitrogen or fixed in 10% of buffered formaldehyde, followed by embedding in paraffin. Sections were examined by immunohistochemical staining for HBsAg expression using HBsAg detection kits (Dako, USA) or by haematoxylin and eosin staining for studying histopathological changes. Tissue sections were read under code by pathologists from two independent laboratories.

2.6. Statistical analysis

The significance of differences between groups was analyzed by paired Student’s t-test.

3. Results

3.1. Serum HBsAg levels

The results are summarized in Fig. 1. The serum HBsAg levels in samples obtained on weeks 0 and 3 were essentially the same for all five groups of animals. In the control unimmunized groups, serum antigen level increased over the 15-weeks period of observation from the mean value of 113 ± 13 to 189 ± 17 ng/ml on week 15 (P < 0.02). In contrast to the controls, the increase of the antigen level was arrested in all immunized groups. In the IC immunized group, compared to the antigen level on week 3, decline in the antigen level was first evidenced on week 12 (P < 0.05) and the antigen sustained at the similar level up to week 15. Immunization with IC–sDNA induced the most marked and rapid decrease in the serum antigen levels. The decline in antigen levels was first evidenced in this group of mice on week 9. Serum HBsAg level was 126 ± 22 ng/ml on week 3 in this group, but declined to 56 ± 14 ng/ml (P < 0.02) on week 9. The decline continued over the subsequent 6 weeks, reaching a low mean level of 28 ng/ml on week 12 and serum HBsAg was sustained at the similarly low level until termination of the experiment on week 15.

3.2. Anti-HBs antibodies

Immunization with IC–sDNA complex elicited the most vigorous antibody response with anti-HBs appearing 3 weeks after the first dose of vaccine (Fig. 2). The antibody rose rapidly after the receipt of the second dose and increased continuously which
Table 2  
IFN-γ induced in HbsAg-stimulated spleen cells  

<table>
<thead>
<tr>
<th>Immunogens</th>
<th>Number of mice</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>HBsAg</td>
<td>6</td>
<td>98 (106)</td>
</tr>
<tr>
<td>IC</td>
<td>6</td>
<td>234 (124)</td>
</tr>
<tr>
<td>IC-sDNA</td>
<td>5</td>
<td>679 (683)</td>
</tr>
<tr>
<td>sDNA</td>
<td>5</td>
<td>132 (52)</td>
</tr>
<tr>
<td>Unimmunized</td>
<td>6</td>
<td>33 (55)</td>
</tr>
</tbody>
</table>

a Average of interferon-γ.

b S.D. of interferon-γ.

extent of reduced expression of HBsAg varied among mice in this group, and was most pronounced in two mice (Fig. 4).

4. Discussion

In a pilot study, we have previously shown that HBsAg complexed to human HBIG (IC) was effective in reducing or clearance of serum HBV viremia in chronic hepatitis B patients [13]. However, no decrease in the serum HBsAg level of the treated patients was observed. When the immunotherapeutic mechanism of this antigen–antibody complex was studied in normal Balb/c mice, we discovered that when plasmid DNA was added to the antigen–antibody complex to generate a new composite, more potent humoral and cellular immune response could be induced [8]. However, when vector plasmid DNA was added to HBsAg-anti-HBs complex to immunize mice, only enhanced anti-HBs

reached $4223 \pm 3301$ mIU/ml on week 15. In IC and HBsAg immunized groups, antibody response was less vigorous, and the levels were $904 \pm 359$ and $149 \pm 149$ mIU/ml, respectively. Antibody response induced by DNA immunization was similar to that elicited by HBsAg alone ($203 \pm 59$ mIU/ml). None of the unimmunized control animals produced detectable level of anti-HBs throughout the course of the experiment.

3.3. Cytokine production

Interferon-γ production from HBsAg-stimulated spleen cells of each immunized group is shown in Table 2. Interferon-γ level varied broadly from mouse to mouse in each group. IC–sDNA elicited a vigorous Th1-type immune response, as shown by the production of the highest level of interferon-γ, and IL-4 production was slightly increased in the IC–sDNA group, which was not of statistical significance (data not shown).

3.4. Cytolytic T cell response

The result of HBV specific CTL activity in all immunized groups are shown in Fig. 3. In IC, DNA, IC–sDNA immunized groups, mouse spleen cells were cytotoxic against Vac–HBsAg recombinant virus infected target cells, but they did not exhibit cytotoxicity against the control vaccinia virus infected cells. CTL response was barely induced in HBsAg immunized mice.

3.5. Histology and expression of HBsAg in liver

No histopathological changes in the liver or kidney from all groups of animals were observed. By immunohistochemical staining, except for the mice immunized with IC–sDNA, expression of HBsAg in liver tissues from the animals was similar to that in the control group. Fewer HBsAg positive hepatocytes were found in liver sections from IC–sDNA immunized mice. The
Fig. 4. Immunohistochemical staining of HBsAg expressed in liver sections of IC-sDNA immunized and unimmunized control transgenic mice. (A) Liver sections of five transgenic mice which were immunized with IC-sDNA (as indicated in the legend of Fig. 1) intramuscularly for four injections at 3-week intervals, sacrificed at week 15 and were stained for HBsAg by Dako immunohistochemical kit. In short, sections were first stained with goat anti-HBsAg overnight, followed by reacting with rabbit anti-goat biotinylated antibody for 30 min, washed and further reacted with streptavidin-HRP-conjugate for another 30 min and finally, the substrate for horse raddish peroxidase was added. Compared to the unimmunized control mice, in two out of the five immunized mice, HBsAg positive hepatocytes show pronounced decrease, and in the other three mice, less HBsAg positive hepatocytes are observed. NC was liver section from a normal control mouse. (B) Liver sections from six unimmunized control transgenic mice.
response was observed; whereas when recombinant plasmid DNA harboring HBsAg gene was added to the complex, both humoral and cell-mediated immune response were enhanced. These results suggested that HBsAg-anti-HBs-DNA complex could be used as a new approach to treat HBV carriage and the chronic disease associated with it. To test this possibility and to compare the efficacy of this composite with other described immunotherapeutic vaccine candidates, we used four immunogens to immunize the same lineage of transgenic mice. The immunization schedule, route and volume of inoculation, anesthetization of animals and genders of mice distributed in each immunized group were designed to minimize bias in results obtained.

In the lineage of HBV-transgenic mice used in this study, HBsAg was expressed by virtually all the hepatocytes and the antigen was detected in increasing concentrations in the consecutive serum samples taken over the 15-weeks duration of our experiment. Presumably, this may be because the rate of antigen production exceeded the rate of disposal, such that there is a tendency for the antigen to accumulate as the animal aged. Though we did not succeed to clear the serum HBsAg or eliminate HBsAg expression in hepatocytes, the pronounced reduction of HBsAg expression in IC-sDNA immunized mice was encouraging.

In this study, even the protein vaccine was able to break the immune tolerance and induced a weak HBV specific immune response in these animals. The immune response induced by s-DNA immunization in this study was not as pronounced as that reported by others [14], which could be due to a different construction of the recombinant plasmid or due to different mouse strain used. However, naked DNA immunization did induce CTL response, production of interferon-γ and anti-HBs, which were adequate to arrest the increase of serum antigen level in animals. The immune response induced by s-DNA immunization in this study was not as pronounced as that reported by others [14], which could be due to a different construction of the recombinant plasmid or due to different mouse strain used. However, naked DNA immunization did induce CTL response, production of interferon-γ and anti-HBs, which were adequate to arrest the increase of serum antigen level in animals. The IC immunogen induced an effective but moderate immune response, which was shown by good CTL response, high interferon-γ production, anti-HBs response and a decline in serum antigen level. IC-sDNA immunization resulted in the best effective response, by marked decrease of serum HBsAg, inducing high level of interferon-γ, high titer of anti-HBs and effective CTL activity. However, in most of the animals, the decrease in serum HBsAg level was not well correlated with the expression of HBsAg in liver tissues. This discrepancy strongly suggest that the decrease in serum HBsAg was mainly due to the neutralizing effect of induced anti-HBs, which was not effective in clearing the HBsAg in hepatocytes.

Only in sections of the liver tissues from IC-sDNA immunized mice, fewer HBsAg positive cells were found. In chimpanzees, a noncytopathogenic antiviral mechanism was described and cytokines played important roles [15]. Due to technical problems, we did not succeed in assaying the HBsAg mRNA in these liver tissues. However, since the level of interferon-γ induced in splenocytes was the highest in this group of mice, the down-regulation of HBsAg expression could possibly be mediated via cytokines, e.g., interferon-γ.

The in vitro cytolytic activity was not observed in liver tissue sections, which could be due to lack of effector cells in the liver tissue of transgenic mice. By haemotoxyn eosin staining, very few mononuclear cells were found in the liver tissues of immunized and control transgenic mice. In addition, the target cells were different between in vitro and in vivo. The hepatocytes expressing the transgene (HBsAg) as targets in vivo could react differently from the recombinant Vac–HBsAg virus infected splenocytes in vitro.

We have shown that by IC immunization, enhanced uptake of HBsAg via the Fc receptors on macrophages and dendritic cells occurred and potentiated in vitro specific lymphocyte proliferation, possibly through the modulated presentation of HBsAg by professional antigen presenting cells [16]. We speculated that when IC–DNA composite was used for intramuscular injection, the professional APCs drawn by IC to the site of inoculation would provide an excellent micro-environment for naked DNA to contact and interact with APC, and presumably, when IC and DNA were co-ingested and processed, the combination of both exogenous and endogenous pathways of antigen presentation could induce potent host immune responses. In addition, the naked DNA in this composite could be protected from enzyme-mediated degradation and be stabilized, and the CpGs in plasmid DNA could serve as the adjuvant to enhance the immunogenicity of the complex [17]. More studies on the immune mechanisms of this composite will elucidate the synergistic therapeutic effects in the transgenic mice model. Since different lineage of mice used and different constructs of immunogens employed could influence the outcome of immunotherapeutic studies, IC-sDNA immunization should be studied in other transgenic mice models, especially in those with active virus replication.

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