Monoclonal antibody against non-dominant epitopes of HBV e Ag was raised by antigen–antibody co-immunization

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Abstract

Detection of hepatitis B e antigen (HBeAg) in the sera of individuals infected with hepatitis B virus (HBV) can indicate both a high infectivity of the disease and a poor prognosis of disease treatment. Most of monoclonal antibodies raised against HBV e proteins interact with immuno-dominant epitopes, such as HBeAg-\textsuperscript{1}/H9252. In order to raise antibodies against non-dominant epitopes of HBV e protein, in this study, mice were immunized with both recombinant HBeAg (rHBeAg) and an anti-HBeAg antibody (EWB) recognizing a dominant antigenic epitope of HBeAg (HBeAg-\textsuperscript{1}/H9252 epitope). With this strategy, we successfully selected two monoclonal antibodies, S-29-3 and S-72-3. Both S-29-3 and S-72-3 bind to recombinant HBeAg with a high affinity. The epitope mapping assay determined that the S-73-2 recognizes the N-terminal of HBeAg (1–118 aa) and the S-29-3 recognizes the C-terminal of HBeAg (91–149 aa). Further experiment showed that these two antibodies could be formed a pair-Abs that is used in detecting native HBeAg from the sera of HBV patients. The conclusion is that the developed method is useful to raise mAb against non-dominant epitopes in given Ag.

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1. Introduction

Hepatitis B virus (HBV) infects more than 300 million people worldwide (Beasley et al., 1981). It is a small DNA virus and consists of a lipid envelope embedded with surface antigen (sAg), HBeAg and the core antigen (cAg) as well as a DNA polymerase (Brunello et al., 1979).

Although it is not clear whether HBeAg plays a role in the pathogenesis of HBV, it has a great value in clinical diagnosis (Klein et al., 1991; Niermeijer et al., 1980). HBeAg is considered as a good marker for indicating HBV infection (Chen et al., 2006; Pal et al., 2005). In nature, HBeAg is always present in a non-particulate state in the serum of HBV-infected individuals (Pal et al., 2005; Takahashi et al., 1983; Magnius and Espmark, 1972). It can be used as a marker of infectivity and diagnosis of patients who have a high risk in transmission of the disease (Okada et al., 1976; Alter et al., 1976). During convalescence, antibodies to HBeAg and HBcAg are detected along with antibodies against the sAg. These antibodies are important serological markers to assess the immunity and the reduction of viral replication in the infected individual (Okada et al., 1976; Alter et al., 1976).

Based on literature, three major epitopes of HBV e protein have been described. They are HBeAg-\textsuperscript{1}, HBeAg-\textsuperscript{1} and HBeAg-\textsuperscript{2} (Sallberg et al., 1991, 1993; Salfeld et al., 1989; Baumeister et al., 2000). HBeAg-\textsuperscript{1} and HBeAg-\textsuperscript{1} are linear epitopes, located on the 128–133 aa and 76–89 aa of the HBeAg, respectively. HBeAg-\textsuperscript{2} is a conformational epitope, covering the total length...
of the HBeAg (2–140 aa). Further studies show that HBeAg-B is a predominant epitope (Sallberg et al., 1991; Salfeld et al., 1989; Baumeister et al., 2000; Ferns and Tedder, 1986).

In most cases, following the immunization with recombinant HBeAg (rHBeAg), the antibodies are mainly against HBeAg-B epitope. To select mAbs recognizing different epitopes that could form a pair-Abs in detection of HBeAg has been proven to be difficult.

Since it is essential to have a pair-Abs recognizing a different epitopes and then to be formed a ELISA-kit in detecting a given Ag, in this study, HBV e protein was selected as an model system based on its available reagents and epitopes’ information. To address our question, an antigen–antibody complex was used for co-immunization. Specifically, mice were immunized with an antigen–antibody complex containing the rHBeAg and the monoclonal antibody (EWB). The latter recognizes the HBeAg-B epitope, 128–133 amino acids. We hypothesized that adding of EWB may decrease the response to HBeAg-B epitope and but selectively enhance the immune response to other epitopes within HBeAg. Here we report that with this strategy, we successfully obtained two anti-HBeAg monoclonal antibodies, S-29-3 and S-72-3. Both of them showed a good specificity and affinity to rHBeAg. The S-73-2 recognizes the fragment of HBeAg (1–118 aa) and the S-29-3 recognizes the fragment of HBeAg (91–149 aa). Using these two mAbs, a sandwich ELISA-kit was developed and it has a potential in clinical diagnosis.

2. Materials and methods

2.1. Antigen and reagents

Recombinant HBeAg, cDNA of HBV e Ag and two commercialized monoclonal antibodies (EWB and LPA), were kindly provided by Kehua Bioengineering (Shanghai) Co. Ltd. LN 18 peptide (119–136 aa, LVSFGWIRTPPAYRPN of HBeAg-B epitope) and NN16 peptide (75–90 aa, NLEDPAS-RDLVSVSYVN of HBeAg-1 epitope) were synthesized using a conventional solid-phase chemical method and purified with HPLC in GL Bio-Chem (Shanghai) Ltd. Bovine serum albumin (BSA) served as a carrier protein. Peroxidase labeled goat anti-mouse IgG were purchased from Takara. T4 DNA ligase was purchased from Promega Company. IPTG, PMSF, and Taq DNA polymerase were from Promega Company.

2.2. Animals

BALB/c mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Animals were kept in conventional conditions and were handled in compliance with Chinese Academy of Sciences guidelines for Animal Care and Use.

2.3. Expression and purification of rHBeAg fragments

Four fragments of rHBeAg (E1, E2, E3, E4) gene were inserted into vector pGEX-4T-1 and transformed into E. coli strain, BL21 (DE3). The bacterial cells were induced by IPTG (0.1 mM) at 30 °C for 4 h in tryptone-phosphate medium (Ying Lin et al., 2004). The cells were pelleted by centrifugation and resuspended. The cell lysates were prepared by sonication. Extracted proteins were purified by column purification with GST sepharose 4B beads (Smith and Johnson, 1988).

2.4. Immunization and monoclonal antibodies raising

Female BALB/c mice were immunized subcutaneously with 0.1 mg rHBeAg and excessive EWB mAb in emulsion 1:1 (v/v) complete Freund’s adjuvant and boosted two times at 3-week interval in incomplete Freund’s adjuvant. The mouse with the highest antibody titer was intraperitoneally boosted with 0.1 mg of the rHBeAg in 0.5 ml PBS. On the third day after the last boosting, the mouse was sacrificed and the spleen cells were harvested (Shang et al., 2005). The spleen cells were fused with murine myeloma cells (SP2/0) by 50% PEG. The hybridomas were generated through the selection of HAT medium. The supernatants of hybridoma cultures from each well was screened by detecting its binding activity to rHBeAg in ELISA. The positive hybridomas cultures were cloned by a limiting dilution. After three cycles of cloning, the stable hybridoma clones were obtained. Antibodies’ isotype was tested by mouse sub-isotype panel (Bio-Rad).

2.5. HRP enzyme labeling purified monoclonal antibodies

The pristine-primed BALB/c mice were injected intraperitoneally with 1 × 10^8 hybridoma cells per mouse. The ascites fluids were collected and purified by protein G affinity column. The purified antibodies were then labeled with horseradish peroxidase (HRP) by two-step method with glutaraldehyde (Shang et al., 2005).

2.6. Measurement of antibody affinity constant

The antibody affinity constant was measured by BIAcore 3000. In brief, HBeAg was first immobilized to CM5 sensory chip. Various concentration (4, 2, 1, 0.5, 0.25, 0.125, 0 μM) of anti-HBeAg monoclonal antibodies was then added to the chip to measure the kinetic constant according to the binding model. The equilibrium dissociation constant K_D was estimated using the formula: K_D = k_d/k_a (Canziani et al., 2004; Yao et al., 2006). The affinity constant K was calculated using the formula: K = 1/k_D. The system buffer used in this assay contains 20 mM HEPES, 150 mM NaCl, 5 mM EDTA and 0.1% P20.

2.7. Western blotting

rHBeAg, E1, E2, E3, E4, LN18-BSA and NN16-BSA were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred onto a...
nitrocellulose membrane (Bio-Rad). After blocking with 3% BSA for 2 h, the membrane was incubated with anti-HBeAg mAbs for 2 h and was then stained with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h. The blots were developed using ECL detection reagents (Amersham Pharmacia Biotech) (Shang et al., 2005).

2.8. Dot blotting

rHBeAg and synthetic peptides LN18 and NN16 were directly added onto the nitrocellulose membrane. After blocking with 3% BSA for 1 h, the membrane was incubated with anti-HBeAg antibodies for 2 h at room temperature and stained with HRPC-conjugated goat anti-mouse IgG antibody for 1 h. ECL-detection reagents (Amersham Pharmacia Biotech) were then used to develop the films.

2.9. Sandwich ELISA assay

Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (Shang et al., 2005). In brief, 96-well microtiter plates were coated with the antibodies in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After being blocked with phosphate-buffered saline (PBS) containing 10% bovine serum and 0.1% Tween 20, the plates were incubated with rHBeAg at 37°C for 2 h. The HRP-coupled anti-HBeAg antibodies were then added to the wells and incubated at 37°C for 1 h. Tetramethylbenzidine (TMB)–H2O2 was used as the substrate of HRP, and the absorbance was measured by microplate autoreader (Thermo) at 450 nm.

3. Results

3.1. Mapping epitope of EWB and LPA

Since EWB and LPA are two anti-HBeAg antibodies, it is necessary to map their epitopes on the HBeAg. Based on the literature, LN18 and NN16 peptides represented the two major linear epitopes of HBeAg-ß/H9252 and HBeAg-1, respectively (Salberg et al., 1991; Salfeld et al., 1989; Baumeister et al., 2000; Ferns and Tedder, 1986). In order to detect whether the two mAbs could bind to those two peptides, the ELISA was performed. The ELISA results showed that both antibodies bind with high specificity to rHBeAg. In addition, EWB binds to the LN18 peptide (HBeAg-ß epitope), but it does not bind to NN16 peptide; while LPA does not react with both of peptides (Fig. 1A).

To further exclude the influence of BSA, the peptides (LN18 and NN16) were directly added onto the nitrocellulose membrane and the dot blotting was performed to detect their binding activity to the mAbs. The results were quite similar to those by ELISA (Fig. 1B), indicating that EWB recognizes the predominant epitope, HBeAg-ß.

3.2. The monoclonal antibodies recognizing non-dominant epitope of HBeAg were generated

In our primary experiments, we have observed that the mAbs sharing the similar epitope recognized by EWB (HBeAg-ß epitope) are preferable to be generated after rHBeAg was used as an antigen to immunize mice. We assumed that HBeAg-ß epitope is a dominant epitope in rHBeAg, during the immunization with the rHBeAg, the Abs recognizing other non-dominant epitopes have been suppressed. To overcome this obstacle, EWB was selected and the mice were co-immunized with both of EWB and rHBeAg to induce antibodies recognizing non-dominant epitopes of rHBeAg. After fusion and screening process, two mAb clones S-29-3 and S-72-3 were selected. The isotypes of the mAbs were determined (Table 1 and Fig. 2A). The results show

![Fig. 1. Epitope mapping of EWB and LPA. (A) Synthetic peptides and rHBeAg were used in epitope mapping of EWB and LPA. Antigens were coated in the plate at the concentration of 2 μg/ml. To block the reactivity between the antibody and the peptides, the antibody was incubated with enough rHBeAg at room temperature for 2 h before adding it to the wells. The binding activity of mAb to Ag was tested by ELISA. (B) Dot blotting assay to map epitope of EWB and LPA. The peptide LN18 and NN16 (5 μg) were added to the NC membrane and then they were detected with mAbs.](image-url)

<table>
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<tr>
<th>Clone</th>
<th>Ab titer (OD450 ± S.D.)</th>
<th>Ig subclass</th>
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<tr>
<td>S-29-3</td>
<td>3.1065 ± 0.06461</td>
<td>IgG1 k</td>
</tr>
<tr>
<td>S-72-3</td>
<td>2.248 ± 0.143</td>
<td>IgG2b k</td>
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* The supernatants were from the culture of 1 × 10⁶ hybridoma cells in 9 ml medium for 3 days and were used directly to detect values of OD450 and isotypes by ELISA.

* Results of ELISA.
that these antibodies have a highly binding activity to rHBeAg and they belong to IgG1 k and IgG2b k, respectively.

To further understand the characteristics of the mAbs, the nature and denature forms of rHBeAg were used to evaluate linear or conformational epitopes recognizing by mAbs. Their affinities to rHBeAg were also measured by BIAcore 3000. The results show that both S-72-3 and S-29-3 bind to the nature form, but not to the denature form of rHBeAg (Table 2 and Fig. 2B); whereas EWB recognizes both forms. In contrast, LPA binds only to the nature form. In the denature experiments, S-72-3 and S-29-3 mAbs lost their binding activity over 80%, whereas the EWB mAb only lost 43% of binding activity. Our data indicated that S-29-3, S-72-3 and LPA might recognize conformational epitopes; while EWB recognizes linear epitope (HBeAg-β epitope). In general, that the binding activity loss over 50% is

<table>
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<th>Antibody</th>
<th>Nature rHBeAg (OD&lt;sub&gt;450&lt;/sub&gt; ± S.D.)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Denature rHBeAg (OD&lt;sub&gt;450&lt;/sub&gt; ± S.D.)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Loss of activity&lt;sup&gt;c&lt;/sup&gt; (%)</th>
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<tbody>
<tr>
<td>S-72-3</td>
<td>2.425 ± 0.0857</td>
<td>0.269 ± 0.0133</td>
<td>89</td>
</tr>
<tr>
<td>S-29-3</td>
<td>1.674 ± 0.0555</td>
<td>0.327 ± 0.0116</td>
<td>80</td>
</tr>
<tr>
<td>LPA</td>
<td>2.011 ± 0.0711</td>
<td>0.355 ± 0.01</td>
<td>83</td>
</tr>
<tr>
<td>EWB</td>
<td>2.924 ± 0.0827</td>
<td>1.652 ± 0.0935</td>
<td>43</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results of ELISA.

<sup>b</sup> By heat-denature method: 95 °C for 5 min.

<sup>c</sup> When the reactivity of an antibody loses over 50% with denature form of antigen, it is considered to be a conformational epitope recognized by a given mAb.

Fig. 2. Characterization of the anti-HBeAg monoclonal antibodies. (A) BSA and rHBeAg were coated on a 96-well ELISA plate at the concentration of 2 µg/ml. After blocking with 10% bovine serum, the plate was incubated with S-29-3 and S-72-3. Then bound antibodies were detected with HRP-conjugated goat anti-mouse IgG antibody and the OD values were measured at 450 nm. (B) Anti-HBeAg antibodies react with nature and denature form of rHBeAg. The proteins were heat-denature and were coated at the same concentration as the nature form. (C) Measurement of antibody affinity constant among four antibodies by BIAcore. The concentration of the antibodies used in this assay ranging from 4, 2, 1, 0.5, 0.25, 0.125, 0 µM. Black line: antibody S-29-3, the affinity constant is 1.59 × 10<sup>8</sup> l/mol. Green line: antibody EWB, the affinity constant is 8.62 × 10<sup>8</sup> l/mol. Red line: antibody S-72-3, the affinity constant is 4.59 × 10<sup>7</sup> l/mol. Blue line: antibody LPA, the affinity constant is 5.52 × 10<sup>6</sup> l/mol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
considered to be conformational epitopes (Baumeister et al., 2000).

When Abs' affinities to rHBeAg were compared among four mAbs, the values of affinity constant for S-29-3 and S-72-3 are $8.62 \times 10^8$ and $4.59 \times 10^7$ l/mol, respectively (Fig. 2C). The affinity constants for commercialized antibodies (EWB and LPA) are $1.59 \times 10^8$ and $5.52 \times 10^6$ l/mol, respectively, suggesting that the affinity of S-29-3 and S-72-3 mAbs is as good as commercialized antibodies (EWB and LPA).

Therefore, all the above data indicate that the mAbs (S-72-3 and S-29-3) raised by an Ag and Ab co-immunization might recognize conformational epitopes and have high affinities to rHBeAg.

### 3.3. Mapping epitope of the anti-HBeAg antibodies

When a pair-Ab is constructed as sandwich ELISA-kit, it is essential for them to recognize different epitopes in the same antigen. In order to map the epitopes recognized by the raised mAbs, full length HBeAg (rHBeAg), four fragments of HBeAg (E1, E2, E3 and E4) and two peptides (LN18 and NN16) were selected (Fig. 3A). The immuno-reactivity between the Ags and the mAbs was determined by Western blotting. The data showed that the S-72-3 and S-29-3 recognized different epitopes (Fig. 3B and C). Both antibodies recognize the full length rHBeAg, but S-29-3 recognizes LN18-BSA and the E3 fragment, but not other three fragments, and thus the antibody S-29-3 recognized the...
region of amino acids 91–149 (including the HBeAg-B epitope) of the HBeAg. In contrast, S-72-3 only recognizes the E4 fragment, which is located at the region of amino acids 1–118, but not any other fragments of the rHBeAg. Our data demonstrated that S-72-3 and S-29-3 mAbs recognize different epitopes. As we expected, when mice were co-immunized with both of EWB (recognizing HBeAg-B epitope) and rHBeAg, the antibodies recognizing non-dominant epitopes of rHBeAg can be induced preferably.

3.4. The two anti-HBe antibodies were composed as a pair-Abs for the detection of HBeAg

Since the S-72-3 and S-29-3 recognize different epitopes, they have a potential to be constructed as a pair-Abs in detection of HBeAg. To verify this possibility, both S-72-3 and S-29-3 mAbs were purified and labeled with HRP enzyme. The different sandwich ELISA assays were performed with them (Fig. 4A). The results showed that both antibodies could be used as coating antibody in detecting rHBeAg and the S-29-3 and S-72-3-HRP pair shows a higher binding activity.

In the following experiments, the sensitivity of the pair-Abs was tested by sandwich ELISA in serial dilution of rHBeAg (Fig. 4B). The limiting detection by ELISA was measured. The results show that its detection level is 2 ng/ml (S-29-3 and S-72-3-HRP pair). Finally, we evaluated the sandwich ELISA by detecting HBeAg in the sera from HBV patients. The sera were obtained from hospitalized patients in Shanghai ChangZheng Hospital by a legal and ethical way. The patients were confirmed with clinical syndromes and serological diagnosis by a commercial HBeAg detecting kit (Murex HBeAg/anti-HBe from ABBOTT). The normal serum samples were from health individuals when they have a physical examination in the hospital. Five HBV patients’ sera (marked as A–E in the paper) and normal sera were used to evaluated the sandwich ELISA we constructed. The results show that the S-29-3 and S-72-3-HRP pair-Abs is much better than the other pair. It can be used to detect HBeAg in sera (Fig. 4C). Taken together, the pair-Abs (S-29-3 and S-72-3-HRP) can be specifically used to detect both rHBeAg and native HBeAg, indicating that the pair-Abs has a potential used in clinical diagnosis.

Fig. 4. The two anti-HBeAg antibodies were composed as pair-Abs for the detection of rHBeAg and native HBeAg. (A) Antibodies were coated at the concentration of 10 μg/ml. After blocking with 10% bovine serum, the plate was incubated with 1 μg/ml rHBeAg. Then HRP labeled antibodies were added with 1:5000 dilution. The absorbance was measured at 450 nm. (B) Compare the sensitivity of pair-Abs by sandwich ELISA. The antibodies were coated at the concentration of 10 μg/ml. rHBeAg was first added at 1 μg/ml and serial dilutions of rHBeAg was detected by HRP-mAbs (1:5000 dilution). The concentration of the rHBeAg is 2^{(-n)} μg/ml. (C) Five HBeAg positive HBV patients’ sera (A–E) were used to evaluate the sandwich ELISA kits we constructed. The negative sample is normal serum sample. The positive sample is normal serum sample added with 1 μg/ml of recombinant HBeAg.
4. Discussion

HBeAg is considered as a good marker of HBV viral blood infection. It can be used as a marker of infectivity and it reflects the patient’s status at high risk for transmission of the disease (Takahashi et al., 1983; Magnius and Espmark, 1972; Okada et al., 1976; Alter et al., 1976). During convalescence, antibodies against HBeAg are important serological marker used to assess immunity and the reduction of viral replication in the infected individual.

To raise antibody against the HBeAg, the initially consideration is to immunize the mice with native HBeAg and select the right clone. But native HBeAg is usually not easy to be separated. In most case, the recombinant HBeAg (rHBeAg) is used as a substitute (Inada et al., 1989). In our previous work, we also observed that in some experiments raised mAbs often binds to a similar epitope in given Ag and it is not easy to have a pair-Ab recognizing a different epitope.

In this study, in order to make sure the mAbs recognizing different epitopes, we tried to improve our protocol of immunization. We used the rHBeAg and an anti-HBeAg antibody (EWB) mixture as the immunogen. In which EWB is a commercially available antibody which recognizes the linear dominant epitope of HBeAg named HBeAg-β. Among the epitopes of HBeAg, HBeAg-β is believed as a dominant one (Sallberg et al., 1991, 1993). Our initial experiments demonstrated that it is very difficult to raise antibodies recognizing epitopes other than HBeAg-β. We expected that the immunization to suppress an immune response specific to a dominant epitope could induce Abs recognizing non-dominant epitopes in a giving Ag.

With this strategy, the rHBeAg and a monoclonal antibody-EWB (it recognizes the HBeAg-β epitope, 128–133 amino acids) were used to co-immunize mouse. Two monoclonal antibodies named S-72-3 and S-29-3 were selected. They bind to rHBeAg with a high affinity and both mAbs recognize conformational epitopes on rHBeAg.

The epitope mapping indicates that S-29-3 and S-72-3 recognize different epitopes. S-29-3 recognizes C-terminal of the rHBeAg (a region of amino acids 91–149) and S-72-3 recognizes the N-terminal of the rHBeAg (1–118). It is interesting to note that rHBeAg-EWB co-immunization succeeded to raise the mAbs that can bind to other epitopes other than HBeAg-β. When they were used as pair-Abs to detect rHBeAg in a sandwich ELISA assay, we found that the pair-Abs show high specificity and sensitivity. We applied it to detect the HBeAg in the HBeAg positive sera from HBV patients. The results showed that our developed kit has a potential to be used in clinical diagnosis.

In summary, an improved antigen–antibody co-immunization method was developed. It could be applied to raise antibody against a given antigen when it is difficult to generate mAbs recognizing different epitopes in diagnosis purpose. In this paper, two monoclonal antibodies named S-72-3 and S-29-3 were directionally raised with the co-immunization method. They showed a high specificity and affinity to the rHBeAg and recognize different epitopes of HBeAg. The S-29-3 and S-72-3-HRP pair-Abs has a good specificity to detect HBeAg, indicating that it has a potential to be used in clinical diagnosis.

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