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Immunoprecipitation of Recombinant Hepatitis B Virus e Antigen By Monoclonal Antibody

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Abstract

Hepatitis B virus (HBV) infection is strongly associated with hepatocellular carcinoma. The sequence of HBeAg in sera of hepatitis patients and the sequence of the entire core antigen in liver-derived HBVs of hepatoma patients reveal elevated mutation frequencies in the highly evolutionarily conserved regions. There may be a relationship between the regions of antigenic determinants of HBeAg and the regions of elevated mutation frequencies of the liver-derived and naturally occurring HBVs. The antigenic epitopes of HBeAg were studied to explain how HBeAg variants accomplish immunoevasion via escape mutation along the antigenic determinants of HBeAg recognized by T-cell and B-cell. Attempts were made to map antigenic determinants of HBeAg using a monoclonal antibody (MAb 8425). Seven potential epitopes of HBeAg were analysed by hydrophilicity and synthesized as peptides. HBeAg recombinant protein, SV, was tested for specific antigenicity with monoclonal antibody and sera from patients with hepatoma and used as a target protein for binding with monoclonal antibody. The blocking activity of each peptide in the binding of monoclonal antibody to the target protein was measured by Western blot immunodetection and ELISA. No blocking effect, by any of the peptides, was identified by either assay. We conclude that the recombinant HBeAg protein expressed in this study was able to compete with MAb for the binding to the HBeAg but the epitope mapping of HBeAg specific for MAb was not successful.

Keywords: Recombinant Hepatitis B virus e antigen , immunoprecipitation

Introduction

Hepatitis B virus (HBV) affects millions of people worldwide, and HBV infection is associated with acute and chronic hepatitis and cirrhosis. In addition, chronic HBV infection is strongly associated with hepatocellular carcinoma (HCC), the most common cancer in the world

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(Szmuness, 1987; Hollinger, 1990; Beasley, 1988). The presence of HBeAg in the serum of HBV-infected individuals (HBsAg positive) is an important clinical marker of ongoing HBV replication and significant liver damage (Nordenfelt and Andren-Sandberg, 1976; Alter *et al.*, 1976). Seroconversion from HBeAg-positive to anti-HBe antibody positive is suggestive of abrogation of viral replication and remission of liver disease (Hoofnagle, 1983; Realdi *et al.*, 1980). In general, patients who are chronically infected with HBV will show HBeAg in their serum. However, a significant subset of chronically infected patients are anti-HBe antibody positive (Okamoto *et al*., 1990; Miyakawa *et al*., 1985). Several reports have been published describing HBV variants isolated from the sera of these patients (Okamoto et al., 1990; Raimondo *et al*., 1990). The most common finding is that of a mutation in the pre-core gene that leads to the production of replication competent virus that does not manufacture HBeAg (Okamoto *et al.*, 1990; Raimondo *et al.*, 1990). This type of pre-core mutant has been associated with severe liver disease and with chronicity and latency of HBV infection. (Raimondo *et al*., 1990; Burnetto *et al.*, 1989).

HBeAg is a core gene product, of hepatitis B virus gene HBcAg expression, that is produced by infected cells. The precursor of HBeAg is encoded by mRNA which is slightly larger than the mRNA from which HBcAg protein is translated. The HBeAg precursor contains a small extra sequence at its N terminus. This peptide acts as a signal sequence for secretion and mediates translocation of the HBeAg precursor into the lumen of the endoplasmic reticulum (Junker *et al.*, 1987; Ou, *et al.*, 1986). After post-translational processing, the first 19 amino acids of a 29 amino acid signal sequence and a strongly basic C-terminal domain are proteolytically removed, and mature HBeAg is secreted by the cell (Garcia *et al.*, 1988; Standing *et al.*, 1988; Takahashi *et al*., 1983). In addition, there are two secreted forms of HBeAgs with 19 amino acids difference (Fig. 1), the HBeAg-MV and HBeAg-SV, which are produced by post-translational processing of the precore gene product (Robinson, 1990: Crowther *et al.*, 1994). The HBeAg-SV is the major form of secreted HBeAg. These two proteins were recombinantly produced from our laboratory (Hwang et al., 1999). Although HBcAg and HBeAg share 149 amino acid residues, these proteins are very different immunologically. HBcAg has T-cell dependent and independent epitopes which are in part responsible for its extremely high immunogenicity both in mice and HBV-infected patients (Milch and McLachlan, 1986).

HBeAg, in contrast to HBcAg, is immunogenic only in a T-cell dependent epitope and is a relatively poor B-cell immunogen (Gerlich and Heermann, 1991). Although HBcAg and HBeAg have been extensively investigated in the past, many aspects of their immunological similarities and differences remain unclear. For example, HBeAg has unique gene expression features and immunological performance, but the functional aspect of HBeAg in HBV immunopathogenesis required further clarification.

In this study, attempts were made to map antigenic epitopes of HBeAg using mouse MAb (MAb 8425). Epitope mapping was performed with a set of synthetic HBeAg peptides and the recombinant HBeAg (HBeAg-SV) fusion protein. Seven selected peptides were predicted from computer analysis, according to degree of hydrophilicity, and were synthesized for the blocking assays. This study may explain how HBeAg variants accomplish immunoevasion via escape mutation along the antigenic determinants of HBeAg, which are recognized by T-cells or B-cells. The accuracy of computer's prediction on the antigenic epitopes corresponding to the highly hydrophilic regions of amino acid sequences were also tested.

Materials and Methods

Analyses of the Antigenic Determinants and the Hydrophilic Regions of HBeAg

The protein conformational features (Chou and Fasman, 1974) and antigenic determinants (Hopp and Woods, 1981) were predicted from the deduced amino acid sequence of HBeAg by two computer programs, PC/GENE (IntelliGenetics, Inc./GENOFIT SA., Switzerland) and GCG (Genetic Computer Group, Inc., Wisconsin). These programs located the points of greatest local hydrophilicity along the sequences by assigning each amino acid a numerical value (hydrophilicity value) and then, repetitively averaging these values along the peptide chain. The GCG program was obtained through the network system, Academic Sinica, Taiwan, R.O.C.

Western Immunodetection of Sera from Patients with Hepatocellular Carcinoma

The specificities of the MAb 8425 (mouse isotype: IgG1, κ, Chemicon International Inc.) and sera of patients with hepatocellular carcinoma against HBeAg were defined by immunodetection. Plasmid containings PCR-amplified HBeAg-SV gene was constructed in pGEX-5x-1 vector and expressed in frame in *E. coli* DH5α. The induced intracellular glutathione S-transferase (GST) fusion protein of HBeAg-SV was recovered from bacterial lysates by affinity chromatography with glutathione-sepharose beads (Pharmacia Biotech). The purified HBeAg-SV protein was separated by SDS-PAGE using 12% gels. After electrophoresis, proteins on the gels were transferred to nitrocellulose membrane. Duplicate blots were generated by diffusion for 24 hr at room temperature. Immunodetection of HBeAg on nitrocellulose membrane was carried out with alkaline phosphatase goat antibodies and NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indylphosphate) were used as chromogen/substrates.

Competitive Enzyme-linked Immunosorbent Assay (ELISA)

ELISA analysis for antigenic epitope mapping of HBeAg was conducted by competitively blocking the specific binding of MAb to the expressed fusion form of HBeAg using the sequence specific synthetic peptides (11-19 amino acids in length) shown in Fig. 1. ELISA determined the extent of binding of MAb to HBeAg fusion protein immobilized overnight at 4^0C to the bottom of wells of a 96-well plate (1 μg/well). The absorption of MAb with peptides was followed as previously described (Hwang and Li, 1993; Huang *et al.*, 1995). After blocking with 3% bovine serum albumin and washing, the HBeAg-bound antibody was detected by peroxidase-conjugated goat anti-mouse IgG and the oxidation of ortho-phenyldiamine (OPD). The degree of oxidation was measured at 490 nm.

Immunoblocking Assay with Synthetic Peptides at Predefined Regions

Western blot analysis for antigenic epitope mapping of HBeAg was conducted by competitively blocking the specific binding of MAb to the expressed fusion form of HBeAg using the sequence specific synthetic peptides (11-19 amino acids in length) shown in Fig. 1. Each of the synthetic peptides was incubated with MAb, diluted to 1:1000. The mixtures were

incubated and shaken overnight at 4^0C. After a brief peroid centrifugation, the cross-absorbed MAb in the supernatant was tested for immunodetection by Western blots in the nonisotopic Amersham ECL detection system. The loss of reactivity with HBeAg confirmed the location of each antigenic epitope.

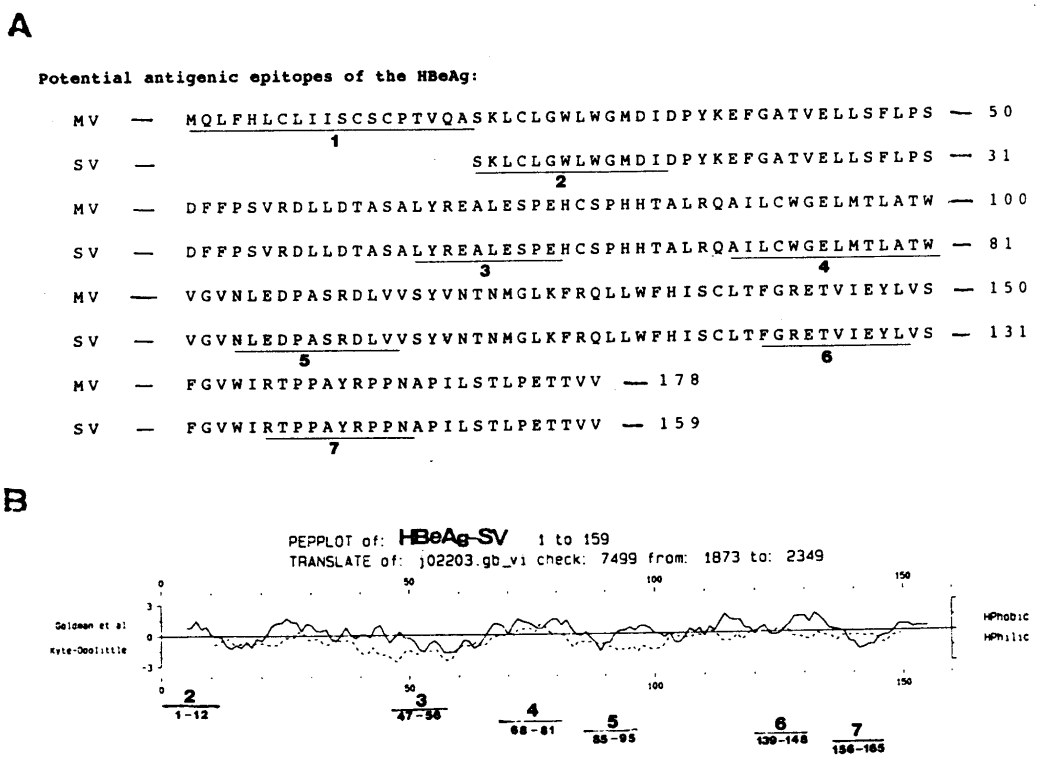

Figure 1. Peptides selected for antigenic epitope mapping. In panel A: amino acid sequence alignments of HBeAg-MV and HBeAg-SV. The seven highlighted peptide (no. 1-7) regions selected for the blocking assay with MAb, were used for determining the antigenic epitopes. In panel B: hydrophilicity and hydrophobicity profiles of HBeAg-SV. The locations of six of the seven synthetic peptides, no. 2-7, used for competitive blocking of MAb to the SV protein are shown.

Results and Discussion

Potential antigenic determinants were identified by analyzing the amino acid sequence ascertained by two computer programs, P/C GENE and GCG. The highly hydrophilic regions of HBeAg detected by these programs are shown in Fig. 1. To determine whether the recombinant SV protein was an appropriate as target protein for blocking assays, the fusion protein HBeAg-SV was tested for its binding ability with the sera from 20 hepatocellular carcinoma patients. The data are shown in Fig. 2. At a human serum dilution of 1:1000, the recombinant HBeAg was bound to sera in 90% of patients, as determined by Western immunodetection. The lower amount of binding to serum in patients no. 16 and no. 17 indicated that these two individuals failed to produce higher titers of anti-HBeAg antibody. A quantitative study of anti-HBeAg titers of sera from 20 patients was also performed by ELISA, as shown in Fig. 3. The HCC patients' sera contained various binding activities to the recombinant HBeAg-SV fusion protein. Results from both studies are corresponding very well. Patients with hepatocellular carcinoma are likely to be anti-HBeAg antibody positive. The data show that the recombinant HBeAg-SV protein was recognized by the sera of patients with hepatocellular carcinoma and also by MAb. This indicated that SV protein can be used as a target protein for blocking assays. Seven predicted antigenic determinants at highly hydrophilic regions, were selected, synthesized, and used as competition reagents for the blocking assays with MAb, for epitopic mapping of HBeAg. The amount of SV protein fully immunoblock the binding of MAb to the target protein was pre-determined to be 500 μg (data not shown). To determine the location of epitope within SV, we used sequence-specific synthetic peptide to block binding between MAb and the target SV protein. At a dilution of 1:1000 of MAb, data from ELISA are shown in Fig. 4. Of the seven synthetic peptides, each 10-19 amino acids long (Fig. 1), none were able to be immunoprecipitated by MAb. Different combinations of synthetic peptides were also used to block binding between MAb and the target SV protein, and data from western blot analyses are shown in Fig. 5. Synthetic peptides with highly hydrophilic sequences, alone or in combination, can not block the binding. In the blocking assay controls, the recombinant HBeAg-SV, which was recognized by both MAb and patients' sera, was used to immunoprecipitate MAb before the supernatant detected the target protein immobilized on the

bottom of the wells of ELISA plates or on the nitrocellulose membranes. The recombinant HBeAg-SV, completely immunoprecipitated by MAb, inhibited the binding of MAb to the target protein as shown in Figs. 4 and 5. The data showed that the seven highly hydrophilic peptides spinning along the HBeAg protein could not react with MAb. Thus, MAb did not recognize any of the seven peptides predicted by the sequence analyses in this study.

Antibodies are generally directed to antigenic determinants or epitopes on the surface of viral proteins (Posnett *et al.*, 1988; Tyler and Field, 1986)). Antigenic determinants that are B-cell epitopes are important in humoral immune response. Antigenic determinants that are T-cell epitopes likely play a role in cellular immune response. Continuous epitopes consist of adjacent residues along the protein chain and are likely to be T-cell epitopes. Discontinuous epitopes consist of nonadjacent residues that structurally interact to form a functional entity, and are usually B-cell epitopes (Rothbard and Gefter, 1991). In this study, seven antigenic determinants of HBeAg were mapped to test for MAb recognition. None of the synthetic peptides, alone or in combination, were able to immunoprecipitate MAb. However, the blocking assays for the positive control could be carried out by ELISA and immunodetection using the recombinant HBeAg-SV. These studies concerning the regions of antigenic determinants, did not locate the specific binding between peptides and MAb. Linear peptides with continuous amino acids spinning in the highly hydrophilic regions of HBeAg could not compete with the binding between HBeAg-SV and MAb. This may be explained by the binding of MAb requiring a conformational structure of HBeAg for recognition. Further studies of epitope mapping require complete overlapping synthetic peptides using multipins, which covered the whole region of HBeAg polypeptide made of 178 amino acid shifting by one amino acid.

In summary, the antigenic determinant of HBeAg is important in humoral immune response. In HCC patients, the host immune response usually does not eradicate the virus from the host. HBeAg and HBsAg variants accomplishing immunoevasion via escape mutation fall within the T-cell epitope types (Hosono *et al.*, 1995 and Tai, *et al.*, 1997). This suggests that the host cellular immune response may play an essential role in interacting with the virus during the period of immunosurveillance.

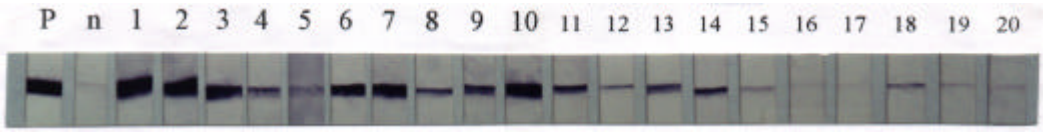

Figure 2. Western immunoblot analysis of sera from 20 patients with hepatocellular carcinoma. SDS-PAGE of the purified fusion protein, SV, SV was immobilized on nitrocellulose paper after Western blotting. Reactivities of sera were detected by immunoblotting using alkaline phosphatase-conjugated goat anti-human IgG (1:1000). Patient serum was diluted 1:1000. Controls including the positive (P, MAb against HBeAg) and negative (N) are shown.

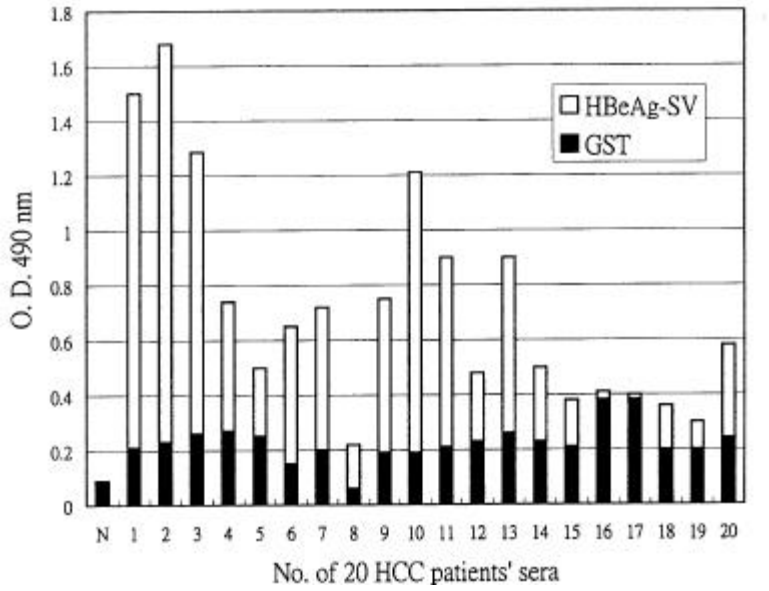

Figure 3. Immunological binding to sera from 20 patients with hepatocellular carcinoma on ELISA. The same amount (25 μg) of purified GST and HBeAg-SV fusion protein were immobilized on the bottom of wells. Reactivity of sera was detected using horseradish peroxidase-conjugated goat anti-human IgG (1:2000). The patients' sera were used in a 1:1000 dilution. N represents the serum from HBV-negative individual.

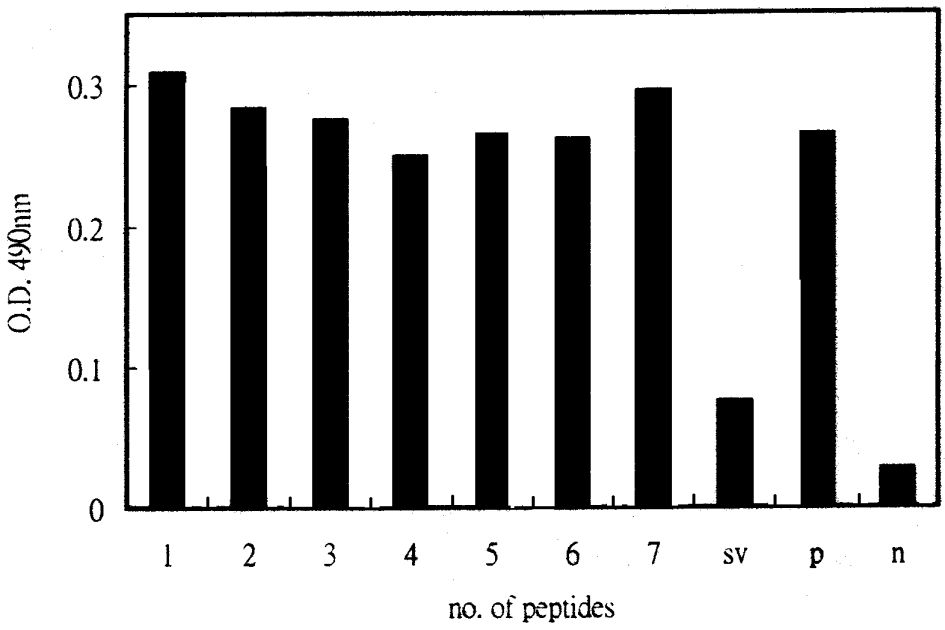

Figure 4. Competition of MAb with sequence-specific synthetic peptides on ELISA. MAb (1:1000 dilution) was independently preabsorbed with 500 μg of each of the seven different synthetic peptides (no. 1-7), and incubated overnight at 4°C, before the supernatant was used for immunodetection of SV coated on the 96-well plate. Controls including the positive (P, no prereaction with SV protein), negative (N, no reaction with MAb), and competition with SV (SV) alone are shown.

A

Peptides \ test tubes	1	2	3	4	5	6	7
1	+	+	+	+	+	+	+
2		+	+	+	+	+	+
3			+	+	+	+	+
4				+	+	+	+
5					+	+	+
6						+	+
7							+

B

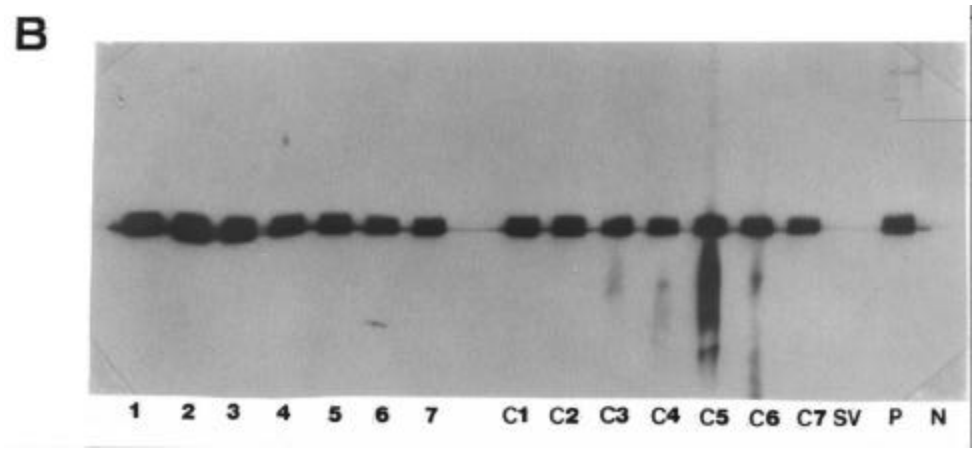

Figure 5. Competition of MAb with sequence-specific synthetic peptides or combination of synthetic peptides (in panel A) on Western blot analysis. The autoradiograph was shown in Panel B. MAb was independently (no. 1-7) or in combination (no. c1-c7) preabsorbed with 500 μg of the seven different synthetic peptides, and incubated overnight at 4℃, before the supernatant was used for immunodetection of SV immobilized on nitrocellulose membrane strips. Controls, including competition with SV protein (SV), positive (P, no prereaction with SV protein), and negative (N, no reaction with MAb), are shown.

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重組 B 型肝炎病毒 e 抗原具與單株抗體免疫沉澱的能力

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摘 要

B 型肝炎病毒感染可能導致肝癌。在慢性 B 型肝炎病人血清中的 e 抗原分子和肝癌病人肝組織取得的核心蛋白分子上有數個演化保留突變區。本實驗的目的是以抗體抗原結合的原理，欲找出 e 抗原分子的抗原決定基部位，比較抗原決定基和保留突變區的相似性，以解釋 B 型肝炎病毒是否藉著突變的方式脫離免疫系統的辨識。介由電腦程式計算得到 e 抗原分子上的 7 段親水性區域，並作成 7 段合成胜太，用合成胜太分別和 e 抗原對單株抗體做免疫競爭結合，以西方點墨免疫測試法和酵素連結免疫吸附測試法，做抗原抗體結合的分析。由基因表達、純化得到的 e 抗原（SV），在定性測試和定量測試中，SV 蛋白和單株抗體有結合反應。另外以 20 位肝癌病人血清測試純化的 SV 蛋白可得到 90% 的 e 抗體反應，表示單株抗體和純化的 SV 蛋白可用於 e 抗原抗原決定基的定位。七段合成胜太分別對單株抗體做競爭結合反應，用酵素連結免疫吸附測試及西方點墨免疫測試法分析結果得知 7 段合成胜太不能與單株抗體做結合反應，然而所表達的重組 e 抗原分子能與單株抗體有免疫沉澱作用。

關鍵詞：重組 B 型肝炎病毒 e 抗原，免疫沉澱力。

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