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行政院國家科學委會專題研究計劃成果報告

抗B型肝炎病毒抗體的選殖及其基因的研究(II)

Characterization of genetic study of anti-hepatitis B virus antibodies (II)

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摘要

如同之前的研究，我們依據基因庫中現存已發表的核甘酸序列,設計並合成一組能放大免疫球蛋白的重鏈以及輕鏈(包括λ和κ)變異區基因的引子.同時,我們也篩選數個正常人血清中存在的可以辨認 HBV 的抗體.聚合酶鏈反應(PCR)之後,可在膠體上出現分子量 680 bp 的主帶,顯示免疫球蛋白基因放大成功.然而,κ鏈基因放大的結果較為一致.大約 90%隨機抽取的細胞株含有輕鏈基因,不過,重鏈基因的插入的效率非常低,結果,我們只得到幾個小的抗體基因庫,每個所含的細胞株都小於 $1x10^5$.因此,我們只好利用這些小的抗體基因庫來篩選任何可能的 anti-HBV 重組嗜菌體.經過 4 次篩選後,超過 50%的隨意分離的細胞株含有 5.0kb 的重組 DNA,這顯示他們都含有重鏈以及輕鏈基因.我們也可以利用點轉澤法(dot blotting)偵測到他們的重組 Fab 分子的表現.不過,他們對純化的 HBsAg 以及 HbcAg 的結合力卻無法以 ELISA 試驗法偵測到.我們目前正在決定這些抗體的重鏈以及輕鏈基因序列.

關鍵字：聯合基因抗體庫；B 型肝炎病毒；Fab 片段

Abstract:

As reported previously, we have designed and synthesized a panel of primers for the amplification of immunoglobulin heavy and light (λ and κ) variable genes based on the published nucleotide sequences deposited in the GeneBank. We also screened the presence of anti-HBsAg antibodies in the sera of several normal individuals. A major band with a molecular weight of 680 bp was detected after polymerase chain reaction, indicating that the Ig genes were successfully amplified. However, the results of κ chain gene amplification is more uniform. Approximately 83% of the randomly isolated clones contains light gene insert. The insertion of heavy chain genes was not highly effective, resulting in several combinatorial antibody libraries with a size of less than $1x10^5$ clones. However, we proceeded the panning of any possible anti-HBV recombinant phage in these small size of antibody libraries. After 4th panning, more than 90% of 15 randomly selected clones contain 5.0 kb recombinant DNA, indicating they have both heavy and light gene inserts. Furthermore, the expression of recombinant Fab molecule was detected in a dot blot. However, the binding activity of these Fab fragments to purified HBsAg and HBcAg was not

seen using ELISA test. We are in the process of determining their gene sequences of heavy and light chains.

Keywords: combinatorial antibody library; hepatitis B virus, Fab fragment

Introduction

Hepatitis B virus (HBV) belongs to the hepadnavirus family, including woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV) and duck hepatitis B virus (DHBV) (Summers et al., 1978; Marion et al., 1980 and 1983). The genome of HBV is circular DNA with 3.2 kilobases in length, which is the smallest animal DNA virus ever found (Yang et al., 1995). The most remarkable feature of this viral genome is its partial double-stranded DNA (dsDNA). HBV infection can cause both acute and chronic necroinflammatory liver diseases and is closely related to the development of primary hepatocellular carcinoma (PHC) (Sherlock et al., 1970). The major source of infectious viral particles is blood, but HBV can be also found in a variety of body fluids including semen, saliva, milk, vaginal and menstrual secretions, and amniotic fluid (Hollinger et al., 1985). The majority of HBV infection in adults result in no significant clinical symptoms, whereas 10% of the acute infected individuals developed chronic liver diseases with various severity. However, more than 90% of neonatally transmitted HBV infections in newborns, spread from infected mothers, become chronically infected (Beasley et al., 1981). As a result, their risk of developing cirrhosis and hepatocellular carcinoma increases dramatically in their life. The pathogenetic mechanisms responsible for the outcome of the HBV infections in different individuals is not fully understood. Up to date, there is no specific treatment for HBV infection, although interferon, interleukin-2, adenine arabinoside, corticosteroids, and acyclovir alone or in certain combinations may be effective in the prevention and control of chronic active hepatitis (Gough, 1983; Saibara et al., 1988; Smith et al., 1983; Bissett et al., 1988; Smith et al., 1982). A great deal of studies suggested that both humoral and cellular immune responses are involved in the viral clearance in the circulation (Chisari and Ferrari, 1995). Cytokines released by virus-activated lymphomononuclear cells may also play an important role in the clearance of the virus.

In this present study, we applied this novel technology to generate and characterize a panel of antibodies against HBV specific antigens, of which antibodies against HBsAg have been shown to protect individuals from HBV infection. We generated a panel of Fab fragments with specific binding activity against hepatitis B virus surface antigen (HBsAg) using the aforementioned combinatorial technique. Taken together, the results from our proposed

studies will further verify whether certain V genes are utilized recurrently to encode both autoantibodies of developmental importance and antibodies against common environmental pathogens. Moreover, the anti-HBV Fab antibodies generated from this study will allow their application in the development of anti-HBV vaccines for the prevention and therapy of acute or chronic HBV infection.

Materials and Methods

RNA preparation and library construction

The library was constructed according to published protocols with minor modifications (Barbas and Burton, 1992). Briefly, total RNA was extracted from 6 x 10^6 peripheral mononuclear cells, and 10 ug of RNAs was reverse-transcribed with an oligo-dT primer to synthesize the first strand cDNA using SuperScript RT kit (Gibco BRL). Then, the λ L chains was amplified by PCR with the CL2 3' primer and 9 different 5' primers for the λ L chain V (Vλ) regions. All primers had been described previously, except for the new VL1B primer (5'-CAG TCT GAG CTC ACT CAG CCA CC-3'; the nucleotides in bold face denote the restriction enzyme site), which was modified from the VL1 primer (5'-AAT TTT GAG CTC ACT CAG CCC CAC-3') in order to amplify Vλ sequences identical to or similar to the L1 IgG RF L chain. Similarly, the γ1 H chain Fd regions was amplified with the CG1Z 3' primer and 8 different 5' VH primers. The PCR consisted of 35 cycles, each consisting of a 15-second denaturation at 94°C, a 50-second priming at 52°C, and a 90-second elongation at 72°C (except for a 10-minute one during the last cycle). Amplified DNA was analyzed on a 2% agarose gel.

Thereafter, the amplified L chain DNA was pooled, digested with SacI and XbaI, and cloned into the SacI/XbaI linearized pComb3 vector. The recombinant DNA was electroporated into XL1-Blue strain Escherichia coli. The size and the insert frequency of the resultant L chain library was determined; then phagemid DNA from the total library was prepared. Thereafter, the amplified H chain Fd region DNA was digested with SpeI and XhoI, and ligated into the SpeI/XhoI linearized phagemid DNA that already contained L chain DNA. The resultant phagemid DNA was electroporated into XL-1 Blue cells as previously. Again, the size and the insert frequency of the resultant L chain-H chain Fd library (designated p3GG) was determined. Phage production was initiated by addition of helper phage VCS-M13, and the recombinant phage was precipitated with 4% polyethylglycerol and 3% NaCl (W/V), resuspended in PBS containing 2% BSA and stored at 4°C until used.

Enrichment and identification of

HBsAg-specific binders

The HBsAg-binding clones in the library was enriched by panning on HBsAg-coated surface. Briefly, four wells in a microtiter plate was coated with 50 ul of commercial available HBsAg at 10 ug/ml in PBS at 4°C overnight, and blocked with 200 ul of 1% BSA in PBS. Then, 10^{11} recombinant phage in PBS with 2% BSA was added to each well, and the plate incubated at 4°C overnight; unbound phage was removed and the wells washed vigorously with Tris-buffered saline (TBS) containing 0.5% Tween-20. Bound phage was eluted with 0.1 M HCl/glycine (pH 2.2)/0.1% BSA, and neutralized with Tris base (pH 9). Eluted phage was used to infect E. Coli; transformed bacteria was mixed with VCS-M13 helper phage, and grown overnight. This panning procedure was repeated 5 more times, with a minor modification that incubation of phage in HBsAg-coated wells was done at 37°C for 2 hours. Thereafter, phagemid DNA from the enriched phage was prepared and digested with NheI and SpeI to remove the phage protein III gene. The digested DNA contains compatible cohesive ends and was self-ligated; the resultant phagemid was electroporated into E. Coli as mentioned above. Individual clones was grown overnight in the presence of isopropyl β-D-thiogalactopyranoside (IPTG). Cells was pelleted and lysed by three cycles of freezing and thawing. After the final thawing, the lysates was centrifuged, and the supernatants was harvested for ELISA assay, while the DNA in the pellet was simultaneously analyzed for H and L chain inserts as described previously.

Enzyme-linked immunosorbent assay (ELISA)

Briefly, microtiter plates was coated with HBsAg molecule (10 ug/ml in PBS) at 4°C overnight. After washing, the plates was blocked with 1% BSA in PBS for 1 hour at 37°C. Then, bacterial lysate or purified Fab fragments was distributed to wells in duplicate and was incubated at 37°C for 1 hour. After washing with PBS with 0.05% Tween 20, the bound Fab fragments was detected with enzyme labeled goat anti-human λ L chain antibody. The degree of oxidation was measured at 492 nm.

Gel electrophoresis and western blotting

Purified Fab antibodies with the HBsAg binding activity was separated by 12% SDS-PAGE under described condition (Laemmli. 1970). After separation, human IgG Fab fragment on the gel was transferred to the nitrocellulose papers by a sandwich-diffusion method. For immunodetection, the membranes was blocked in 1% BSA dissolved in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween-20. pH 8.0) for 1 hr (10 minutes each) at room temperature

and then incubated with properly diluted goat anti-human λ and γ specific antibodies conjugated with alkaline phosphotase for one additional hr. The blot was developed in substrate solution prepared by mixing reagents A and B in buffer as recommended by the manufacturer (Bio-Rad). Color development was stopped simply by rinsing the blot in distilled water.

Determination of nucleotide sequences

The V region nucleotide sequences of 20 randomly chosen RF Fab fragments was determined from double-stranded DNA using dideoxynucleotide termination method (Sanger et al., 1977). The sequencing primers are SEQ Gb (5'-GTC GTT GAC CAG GCA GCC CAG-3') and SEQ T3 (5'-ATT AAC CCT CAC TAA AG-3') for H chain, and SEQ Lb (5'-GAA GTC ACT TAT GAG ACA CAC-3') and KEF (5'-GAA TTC TAA ACT AGC TAG TCG-3') for lambda L chain. The computer programs of the University of Wisconsin Genetics Computer Group (GCG) was used to compile and analyze sequence data.

Results and Discussions

Construction of antibody libraries

We have established at least 6 antibody libraries from the peripheral mononuclear cells isolated from anti-HBV antibody positive individuals. However, all the libraries contain small numbers of clones as indicated in Table I. A library with highest numbers of clones (WHxWLxPH) was used for 4 rounds of panning against HBsAg. As shown in the Figure I, 90% of those selected clones contain a size of approximately 5.0 kb recombinant DNA, suggesting that they have both heavy and light chain gene inserts. To detect the Fab fragment expression in those clones, dot and western blot analysis were performed. The results of dot blot were represented in Figure II. All the clones did show positive reactivity with goat anti-human lambda antibody. A minor band localized at 50 Kd molecule weight in the western blot paper was also detected; however, the reactivity was so weak to show in a duplicated form. We are in the process of concentrating the samples and increasing the titer of goat anti-human lambda antibody using in the western blot analysis. The ELISA result is so far readable, probably also due to the low expression of Fab fragments in the cellular lysates. Preliminary partial DNA sequence data indicated that some of those clones apply the same heavy and light usage, indicating the those clones were specifically enriched and amplified through the panning procedure (data not shown).

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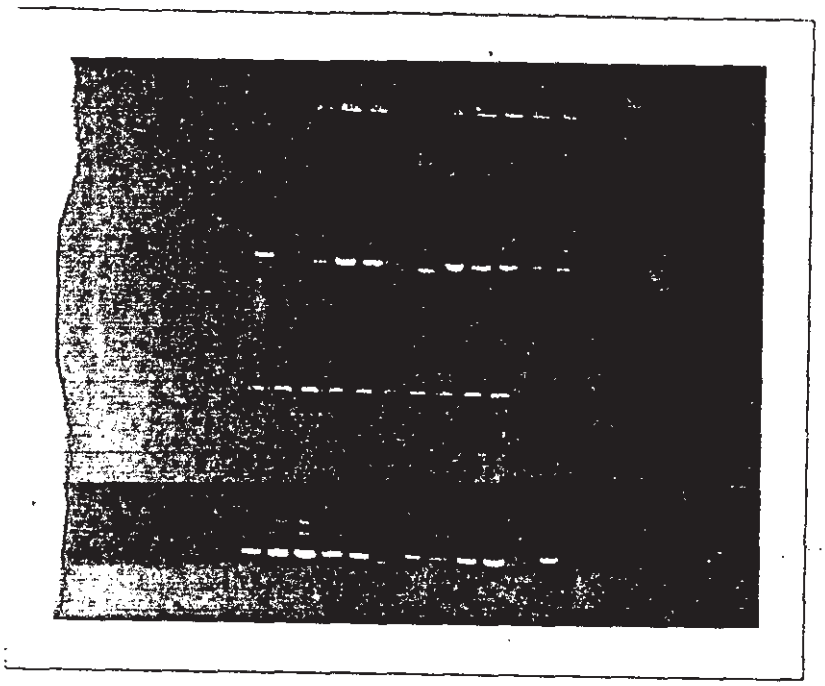

Figure 1. Gel electrophoresis of recombinant DNAs after 4th panning. The individual plasmid DNA was isolated from bacterial clones randomly selected from antibody library after 4th panning and subjected to 1% agarose gel. The DNA was visualized by ethidium bromide.

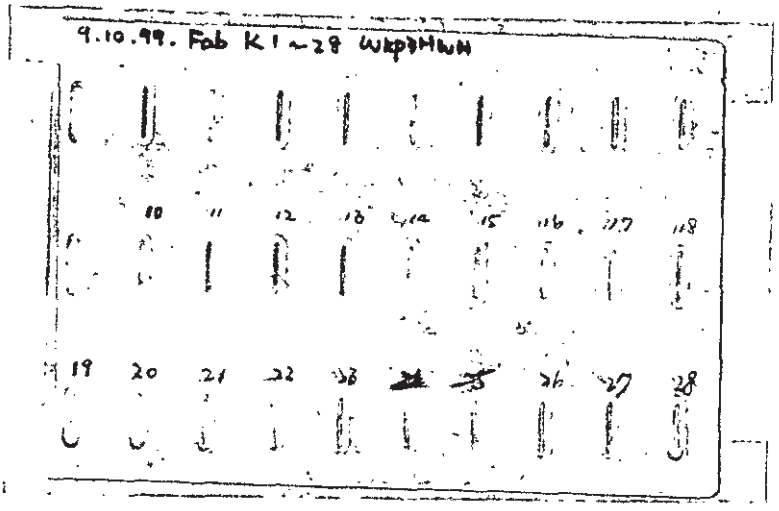

Figure 2. Dot blot analysis of recombinant Fab expressed in E.coli. The bacterial culture was grown to an optical density of 0.6 and the induction of Fab expression was performed by the adding IPTG to a final concentration of 0.1 mM. After overnight , the E.coli cells were pelleted and resuspended in 1x PBS. The cellular lysate was obtained by 3 cycles of freeze (-70°C)and thaw (37°C). Five µl of each sample was loaded onto a nitrocellulose membrane. Goat anti-human lambda or kappa light antibody conjugated with horse radish peroxidase was used as primary probe at 1:3000 dilution.