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行政院國家科學委員會補助專題研究計畫成果報告

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以噬菌體展出系統來篩選C型肝炎病毒特異性的重組人類抗體的研究（I）

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Generation and characterization of recombinant human antibodies specific for hepatitis C virus by phage displaying system **(I)**

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計畫主持人：楊沂淵

共同主持人：

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中文摘要

C型肝炎是由C型肝炎病毒經體液傳染的病毒性肝炎，患者易導致慢性肝炎、肝硬化、甚至肝癌而死亡。據估計目前全世界 C 型肝炎帶原者之人口約超過 10 億，因此如何預防與治療C型肝炎是肝病防治上的一個重要議題。 HCV 為帶有套膜（envelope）的正，單股RNA病毒，長度約 9.5 kb，分類上屬於 *Flaviviridae* 科，HCV 的非結構蛋白 NS3 是一種 immunodominant antigen，具有 serine protease 與 RNA helicase 兩種活性，其基因序列具有 highly conserved，在 HCV 的複製過程中佔有一重要地位。

本實驗是利用 Dr. Toshiaki Maruyama 所構築的 2 個 C 型肝炎抗體基因庫，其大小分別是:含有 kappa 輕鏈與重鏈的基因庫為 1.3×10^7（簡稱為 HCK），含有 lambda 輕鏈與重鏈的基因庫為 2.1×10^6（簡稱為 HCL)。經過嗜菌體的篩選(Biopanning)和富化(Enrichment)，然後利用西方墨點法(Western blotting)由 30 個菌株中各篩選出 15 個具有抗體片段表現的菌株，藉由 ELISA 分析發現 lambda 輕鏈的 2 個菌株對 NS3 抗原具有結合的特異性，不過我們卻無法篩選出任何具有 kappa 輕鏈的 NS3 陽性菌株，以亞培公司所設計的 MEIA 來做確認，發現 HCL4 與 HCL7 菌株對 HCV 抗原呈陽性反應(2.47 S/CO，1.33 S/CO)。進一步分析 20 個 kappa 輕鏈與重鏈及 20 個 lambda 輕鏈與重鏈的 DNA 序列，發現 kappa 輕鏈之 HCK5 菌株的輕鏈在 CDR3 部份的胺基酸序列與 Dr. Maruyama 所找出之 YKns3a1 的輕鏈相同，但重鏈部份則不相同，而 lambda 輕鏈之 HCL4 菌株在重鏈 CDR3 部份的胺基酸序列則與 Dr. Maruyama 的 YKns3b 的重鏈相同，但輕鏈部份則不相同。由以上結果我們得知:重鏈在 anti-NS3 抗體之活性扮演較重要之角色，而利用大腸桿菌來製造和 C 型肝炎 NS3 抗原具有結合能力的人類抗體蛋白片段應是可行的且具有發展的價值。

Abstract

In this study, hepatitis C antibody libraries were established and the heavy chain genes with either kappa or lambda light chain genes resulted in 1.3×10^7 (HCK) and 2.1×10^6 (HCL) clones in size, respectively. After biopanning against the NS3 of HCV, we used Western blot analysis to randomly check 30 clones and found that 15 contained Fab fragment.The preliminary enzyme-linked

immunoabsorbent assay (ELISA) data suggest that 2 clones containing lambda light chain (HCL4 and L7) may be specific for NS3. Furthermore, their NS3 binding activity was confirmed by AxSYM HCV 3.0 Microparticle Enzyme Immunoassay (MEIA). DNA sequence analysis indicated that HCK5 and YKns3a1 contained identical CDR3 region in kappa light chain gene, but differed in the heavy chain. Moreover HCL4 and YKns3b used the same heavy chain, but different light chain genes! Taken together, our results showed that the NS3 binding activity of those screened anti-NS3 Fab antibodies was mainly determined by the heavy chain. The conclusion is consistent with most of the previous studies which indicated that the heavy chain plays an important role in antigen binding activity.

Introduction

Hepatitis C virus (HCV) first identified in 1989 is the major causative agent of parenterally transmitted and community-acquried non-A, non-B hepatitis (NANBH) (Choo et al., 1989). The most common and apparently most efficient route of transmission of HCV is parenteral. HCV is 10 kb-positive, small enveloped RNA virus about 30-38 nm (Yuasa et al., 1991). The virus itself is an RNA virus of about 9,400 nucleotide bases and is now considered a characteristics typical of the family *Flaviviridae*. HCV is organized by a 5'-untranslated region that is highly conserved among strains (Okamoto et al., 1991). This region is followed by an open reading frame coding about 3,010 to 3,011 amino acids. These amino acids are subdivided into nine proteins by order: core (C), envelope (E) E1, E2, nonstructure (NS) NS1, NS2, NS3, NS4 (A and B), and NS5 (A and B) (Takamizawa et al., 1991). These proteins are formed by the cleavage of the original polyprotein by both host and viral proteases (Hijikata et al., 1993). The C region encodes the core nucleocapsid protein (Takeuchi et al., 1990). E1 has been shown to be a glycosylated envelop protein. Whether E2/NS1 represents another envelope protein or a nonstructural one remains controversial (Hahm et al., 1995). NS2 protein is a transmembrane protein, the carboxyl terminal inserts into lumen of endoplasmic reticulum; whereas the amino terminal is localized in the cytosol. The functions of NS2 protein is not clear at this time, but data demonstrated that the carboxyl terminus of NS2 protein have proteolytic function (Santolini et al., 1995). NS3 is a 70 kDa multifunctional protein. The NS3 encodes viral protease that cleaves at least four sites downstream of the viral proteins. Moreover, the NS3 encodes RNA helicase activity, according to protein crystal analysis there are NTPase and RNA binding domains (Yao et al., 1997). The RNA binding domain utilized arginine-rich sequence for recognition of nucleic acid substrate at 3' single-stranded region. Recent work suggested that NS3/4A is a serine protease similar to chymotrypsin and that it helps cleave the original polyprotein (Tanji et al., 1995). NS5 encodes two proteins NS5A and NS5B, and has been demonstrated to exist as a protein comprising

two polypeptides acting as an RNA-dependent RNA polymerase (Major and Friestone 1997). The life cycle of HCV is not clearly understood.

The recent application of immunoglobulin gene combinatorial library technology has led to a logarithmic increase in information concerning human, disease-associated, organ-specific autoantibodies of the IgG class. Many studies have suggested that certain autoantibodies are important at different stages of development and may be instrumental in maintaining homeostasis of the adult antibody repertoire. Unfortunately, only a few heavy and light chain V genes of such antibodies have been characterized due to the difficulties associated with the production of human monospecific antibodies (Jain et al., 1995). To rectify this situation, we propose to apply the newly developed combinatorial antibody technology (Barbas and Burton, 1992), which clones and expresses the Fab fragments of human antibody repertoire on the surface of filamentous M13 phages, to characterize a large number of Vh and V genes. The main power of this new phage display system is the ability to enrich and select a large number of antibodies with desired antigen-binding specificity from a combinatorial library using a novel panning process.

In this present study, we propose to apply this novel technology to construct and characterize a panel of antibodies against HCV NS3 antigen. It has been suggested that NS3 plays a role in pathogenesis, possibly in the development of liver cancer. This may be related to a number of other reports (Borowski et al., 1996, 1997) that the NS3 protein is able to specifically interact with the catalytic subunit of protein kinase A (PKA). PKA is involved in intracellular signal transduction processes and so interference by NS3 is these pathways would be expected to have a dramatic effect on normal cellular functions and would be closely associated with pathogenic mechanisms (Clarke, 1997). Furthermore, NS3 protein is a good therapeutic target molecule for use in developing anti-HCV drugs since its activity is considered to be essential for viral proliferation.

Materials and methods

PCR amplification of HC and LC variable region

Total RNA were extracted from 2 x 10^6 peripheral mononuclear cells by commercial method. Briefly, Trizol buffer (1 ml) and chloroform were added to the tube to lyse the cells. After centrifugation, total RNAs were collected at the aqueous phase, precipitated by isopropyl alcohol, and dissolved in DEPC-treated water. The concentration of RNA samples were measured at OD 260. 1-10 ug of RNAs were reverse-transcribed with an oligo-dT primer to synthesize the first strand cDNA using SuperScript reverse transcriptase.

The λ L chains were amplified by PCR with the CL2 3' primer and 9 different 5' primers. All primers were synthesized as previously described. Similarly, the γ1 H chain Fd regions were amplified with the CG1Z 3' primer and 8 different 5' VH primers . The PCR were performed for 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 during the last cycle). Amplified DNA were analyzed on a 1% agarose gel.

Phage display antibody library construction

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

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated with NS3 antigen (10 ug/ml in 1x PBS) at 4°C overnight. After washing, the plates were blocked with 1% BSA in 1x PBS for 1 hour at 37°C. Then, bacterial lysate or purified Fab fragments were distributed to wells in duplicate and incubated at 37°C for 1 hour. After washing with 1x PBS with 0.05% Tween 20, the bound Fab fragments were detected with enzyme labeled goat anti-human λ L chain antibody.

Results and Discussion

In collaboration with Dr. Toshiaki Maruyama, 2 antibodies libraries containing lambda and kappa light chains, respectively, have been obtained and screened for HCV NS3 specific antibodies using a novel biopanning procedure. After the final panning step, the Fab antibody expression of 15 clones was examined by western blotting and their NS3 binding specificity was confirmed by ELISA and MEIA methods. Among these clones, HCL4 and L7 showed HCV viral protein binding activity compared to that of sera of HCV positive patient. Sequence analysis revealed that heavy chain CDR3 region containing YYCAR DLYIGPTDFYYGMDV WGQG segment is predominant in most of the HCV-positive clones and these clones are represented as LCL4. This result is consistent with that of Dr. Toshiaki in which an independent panning procedure was performed and most clones were found to contain the same amino acid sequences in their heavy chain CDR3 region, indicating that this heavy chain may play an important role in the binding of antibodies to NS3 protein. Taken together, we have successfully cloned 2 anti-HCV NS3 antibodies from phage displaying antibody libraries constructed from a hepatitis C virus infected patient complicated with Sjogren's syndrome.

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