Production and Gene Cloning of Monoclonal Antibodies Directed Against S Antigen of Hepatitis B virus

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We produced two murine monoclonal antibodies designated S2E1 and S2C11, which recognize S antigen of hepatitis B virus (HBsAg). S2E1 could bind to denatured form of recombinant HBsAg as well as native form of HBsAg, but S2C11 could bind only to native form of HBsAg. Both antibodies reacted with HBsAg in the hepatocyte of patient infected with hepatitis B virus. Analyses of the nucleotide sequences encoding the variable regions of these antibodies revealed that S2E1 and S2C11 utilize variable gene segment which belong to Vκ4/5 gene family and utilize the Jκ5 and Jκ4 gene segments, respectively. In addition, the heavy chain of S2E1 express a member of V_H14 gene family and a member of DSP2.9 and J_H3 gene families. S2C11 is related to the V_H1 gene family and expresses DFL16.1 gene regions in conjunction with the J_H3 gene segment. *Korean J. Immunol.* 21, 2: 115~120, 1999

Key Words: Ig variable region gene, Monoclonal antibody, HBsAg

INTRODUCTION

Hepatitis B virus (HBV) is an enveloped DNA virus of the *Hepadnavirus* family which causes acute and chronic hepatitis[1]. Its genome is encapsidated with a virus-encoded polymerase in a 27-nm diameter nucleocapsid surrounded by a host-derived lipid envelope inserted with three viral surface proteins which differ based on the initiation of translation from distinct start codons[1]. In addition to the virion, HBsAg can also be independently secreted from cells as 22-nm diameter spherical or tubular subviral particles which consist of host lipids and viral envelope proteins[1]. Virion and subviral particle envelopes consist predominantly of a glycoprotein of 226 amino acids (small HBsAg) which is primarily responsible for the serotypes of HBsAg[1,2].

A possible role of the small HBsAg in the viral attachment and the virion morphogenesis was suggested. The small HBsAg could bind specifically to intact human hepatocytes[3], and as a specific small HBsAg-binding protein on human liver plasma membrane, apolipoprotein H and endonexin II was identified[4,5]. The species specific distribution of endonexin II apparently correlated with the species tropism of HBV[6]. While, a cytoplasmic domain of the small HBsAg bound to HBV core particles, so the small HBsAg might contribute to the virion morphogenesis[7].

Immune responses to the small HBsAg have been assumed to play an essential role not only in prevention from the HBV-infection but also in the elimination of the virus[1]. HBsAg-vaccination was able to induce the prodution of protective antibodies in greater than 90% of healthy subjects and prevent HBV infection[8]. Antibodies

against the a determinants of the small HBsAg confer protection in adults to all the common subtypes of HBV, whereas antibodies to the subtype determinants do not[9]. The a determinant epitopes are located within domains bordered by amino acids 120 to 147 in all known serotypes of HBV[9]. Antibodies against the small HBsAg were readily detectable in patients who clear the virus and recover from acute hepatitis, but usually not detectable in patients with chronic hepatitis, so they were thought to play a critical role in viral clearance by formation of immune complex with free viral particles and removing them from circulation or possibly by prevention of their attachment to susceptible cells[1]. The small HBsAg was also capable of eliciting CD8+ T cell immune responses[1,10].

Recent technological advances have made it possible to clone variable region genes of Ig and to express of Ig genes in variety kind of cells[11,12]. Furthermore, intracellular expression of antibodies (intracellular Ab) has been reported as a method to inhibit function or expression of the targeted molecule inside a cell[13~16]. In present study, we produced and characterized two monoclonal Abs directed against the small HBsAg and cloned the variable regions of these Abs. The cloned cDNAs of variable regions will be used in the construction of vectors for intracellular Ab in order to block expression of HBsAg and HBV replication.

MATERIALS AND METHODS

Generation of hybridoma cells

Male Balb/c mice (6 to 8 week old) were immunized by intraperitoneal injection of recombinant small HBsAg (Green Cross Co., Korea). For the first immunization, 50 µg of the small HBsAg was emulsified in complete Freund's adjuvant and for the 3 times boosting, 25 µg of antigen was emulsified in incomplete Freund's adjuvant. Three days before cell fusion the mice were given an additional intraperitoneal booster injection of the small HBsAg. Spleen cells from the immunized mice were fused with cells of the myeloma line P3x63Ag8.653 using polyethylene glycol as described[17]. Cell culture supernatants were screened for Abs by ELISA and positive cell lines were subcloned twice by limited dilution.

Immunoglobulin subclass was determined by ImmunoPure Monoclonal Ab Isotyping kit (Pierce) using cell culture supernatant.

ELISA

EIA plates were coated with recombinant small HBsAg

at a concentration of 1 µg per well in carbonate-bicarbonate buffer for 2 h at 37℃. EIA plates were washed three times with 0.1% PBS-Tween 20 and unspecific binding sites were blocked by incubation with 5% BSA-PBS for 1 h. Hybridoma culture supernatant were added and incubated for 1 h. EIA plates were washed three times and then bound Abs were detected with alkaline phosphatase conjugated anti-mouse Ig Abs. After development with substrate solution (1 mg of p-nitrophenyl phosphate per ml in 50 mM sodium carbonate, 2 mM MgCl₂), the optical density was measured at 405 nm.

For examination of reactivity of hybridoma culture supernatant with native HBsAg, ELISA was performed using GENEDIA Anti-HBs ELISA 3.0 (Green Cross Co.). In this ELISA kit plasma-derived HBsAg was used.

Immunoblotting

Recombinant small HBsAg was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters. The nitrocellulose filters were incubated 2 h in PBS supplemented with 5% bovine serum albumin (BSA), incubated for 2 h at room temperature with hybridoma culture supernatant or recombinant small HBsAg-immune mouse sera and washed three times at room temperature in PBS-0.1% Tween 20. The nitrocellulose filters were incubated 2 h at room temperature with alkaline phosphatase conjuated anti-mouse Ig Abs, washed three times as above, and incubated in buffer containing 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate and 0.3 mg/ml of nitro blue tetrazolium.

Immunohistochemistry

Liver tissue was obtained by ultrasonography guided needle aspiration biopsy from the patient diagnosed as chronic B vrial hepatitis. After deparaffinization and rehydration, sections were incubated with a serum free blocking solution (X0909, Dako) for 30 minutes. Then, the sections were incubated overnight at 4℃ with hybridoma cell culture supernatants. After successive washing in PBS the immunohistochemical detection was performed according to the labeled streptavidin-biotin complex method using LSAB kit (DAKO). Sections were finally developed in deaminobenzidine and counterstained by Mayers hematoxylin.

Cloning and sequencing of cDNAs encoding Ig variable regions

The total RNA was extracted from hybridoma cells using acidic guanidine isothiocyanate phenol chloroform extraction method. cDNA was synthesized using Moloney murine leukemia virus-reverse transcriptase (Gibco
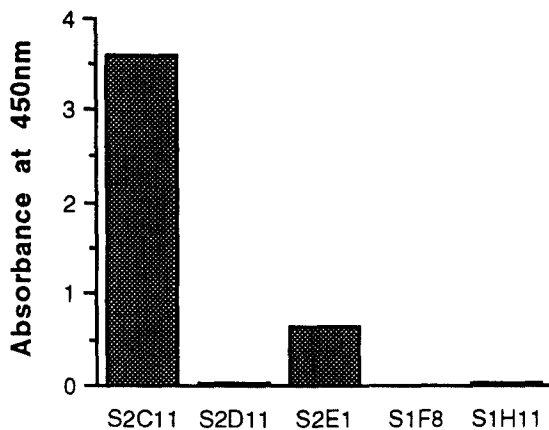
Figure 1. The reactivity of hybridoma culture supernatants to HBsAg. Hybridoma culture supernatants were measured by ELISA for binding to plasma-derived HBsAg.

Figure 3. The reactivity of hybridoma culture supernatants with denatured forms of the small HBsAg. (A) Recombinant small HBsAg were separated by SDS-PAGE and stained with Coomassie blue. (B) Immunoblot of recombinant small HBsAg was performed with preimmune serum (lane 1), small HBsAg-immune mouse serum (lane 2), hybridoma culture supernatant containing S2C11 (lane 3), or hybridoma culture supernatant containing S2E1 (lane 4). M, molecular weight standard.

BRL) and Cκl[18], G1A or G2aA primers. G1A primer was 5'-CTTCTGGGACTGTACATATGC and G2aA primer was 5'-GTTAGGTGCTGGGCATTTGC. The cDNA was utilized in PCR cycles as a template. Degenerate sense primers designed for amplification of mouse V_H and V_L were described previously[18]. Antisense primer for V_H was G1B; 5'-GCTTACAACCACAATCCCT or G2aB; 5'-GAGGAC-AGGGCTTGATTG. The PCR reaction mixture contained 0.5 μl of the cDNA, 5 pmol of each primer, 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 1.2 U DNA polymerase (TaKaRa) was subjected to 30 cycles. The annealing temperature of the reaction is decreased 1°C every cycle from 62°C to 52°C and then 0.5°C from 52°C to 47°C. The PCR products were cloned using the pGEM-T Easy vector system I (Promega). Several clones for each chain were sequenced to ascertain the absence of artifacts arising from PCR amplification. The sequencing was done in an Applied Biosystems PRISM automated sequencer using Big Dye Terminator cycle sequencing Ready Reaction kit (Applied Biosystems).

RESULTS

The reactivity of monoclonal Abs with the small HBsAg

Five hybridoma cell clones producing Abs designated S2C11, S2D11, S2E1, S1F8, or S1H11 were generated. These Abs were reactive with bacterially expressed recombinant small HBsAg. To know these monoclonal Abs could recognize native HBsAg, ELISA using plasma

Figure 2. Immunohistochemical characterization of S2C11 and S2E1. Liver sections from a patient with chronic B viral hepatitis, immunostained with hybridoma culture supernatant containing S2E1 (A) or S2C11 (B), were showing a strong cytoplasmic immunoreactivity in some hepatocytes.

```
Vκ
         1/1                                        31/11
S2C11    atg gat ttt cag gtg cag att atc agc ttc ctg cta atc agt gcc tca gtc ata ata tcc
S2E1     --- --- --a --- --- --- --- t-- --- --- --- --- --- --- --- --- --- --- ---
S2C11    M   D   F   Q   V   Q   I   S   F   L   L   I   S   A   S   V   I   I   S
S2E1     -   -   L   -   -   -   -   F   -   -   -   -   -   -   -   -   -   -   -

         61/21                                      91/31
S2C11    aga gga CAA ATT GTT CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAG AAG
S2E1     --- --- --- --- --- --- --- --- --- --- --- GA- --- --- --- --- --- --- --- ---
S2E1     R   G   Q   I   V   L   T   Q   S   P   A   I   M   S   A   S   P   G   E   K
S2E1     -   -   -   -   -   -   -   -   -   -   -   D   -   -   -   -   -   -   -   -

                             CDR1
         121/41                                     151/51
S2C11    GTC ACC ATG ATC TGC AGT GCC AGC TCA AGT GTA AGT TAC ATG CAC TGG TAC CAG CAG AAG
S2E1     --- --- --- -C- --- --- --- --- --- --- --- --- --- --A --- --- --- --- ---
S2C11    V   T   M   I   C   S   A   S   S   S   V   S   Y   M   H   W   Y   Q   Q   K
S2E1     -   -   -   T   -   -   -   -   -   -   -   -   -   I   -   -   -   -   -

                                         CDR2
         181/61                                     211/71
S2C11    TCA GGC ACC TCC CCC AGA AAA TGG ATT TAT GAC ACA TCC AAA CTG GCT TCT GGA GTC CC
S2E1     --- --- --- --- --- --A -T- --- --- --- --T --- --- --- --- --- --- --- ---
S2C11    S   G   T   S   P   R   K   W   I   Y   D   T   S   K   L   A   S   G   V   P
S2E1     -   -   -   -   -   K   I   -   -   -   -   -   -   -   -   -   -   -   -   -

         241/81                                     271/91
S2C11    ACT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AAT ATG GA
S2E1     G-- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- -GC --- --
S2C11    T   R   F   S   G   S   G   S   G   T   S   Y   S   L   T   I   S   N   M   E
S2E1     A   -   -   -   -   -   -   -   -   -   -   P   -   -   -   -   S   -   -

                                     CDR3
         301/101                                    331/111
S2C11    GCT GAG GAT GCT GCC ACT TAT TAC TGC CAG CAG TGG AGT AGT AAC CCA TTC ACG TTC GGC
S2E1     --- --A --- --- --- --- --- --- --- --- --- --- --- --- -T- --G C-- --- --- ---
S2C11    A   D   D   A   A   T   Y   Y   C   Q   Q   W   S   S   N   P   F   T   F   G
S2E1     -   E   -   -   -   -   -   -   -   -   -   -   -   -   I   -   L   -   -   -

         361/121
S2C11    TCG GGG ACA AAG TTG GAA ATA AAA CGG
S2E1     G-T --- --C --- C-- -CG CTG --- ---
S2C11    S   G   T   K   L   E   I   K   R
S2E1     A   -   -   -   -   A   L   -   -
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Figure 4. The Vκ gene sequences of S2E1 and S2C11. Nucleotides and amino acids homologous to S2C11 are indicated by (−). Nonhomologous nucleotides or amino acids are indicated by their corresponding correct residues. CDRs are underlined. Italic letters and small letters indicate the primer and the leader sequence, respectively.

derived HBsAg was performed. Figure 1 showed that culture supernatant containing S2C11 or S2E1 could bind to native HBsAg. These culture supernatants also showed the strong reactivity with HBsAg expressed in the cytoplasm of HBV infected liver tissue by immunohistochemistry (Figure 2).

The reactivity of culture supernatant containing S2C11 or S2E1 with denatured small HBsAg was examined by immunoblotting (Figure 3) Culture supernatant containing S2E1 reacted with denatured smll HBsAg, but culture supernatant containing S2C11 did not. This finding suggests that S2E1 might recognize linear epitope whereas S2C11 recognize conformation dependent epitope of the small HBsAg.

S2C11 and S2E1 have γ2a and γ1 heavy chains, respectively, and both Abs have κ light chains.

Sequence analysis of Ig variable regions

The variable region cDNA and derived amino acid sequences for S2C11 and S2E1 are shown in Figure 4 and

```
(A) S2E1Vн
     1/1                                       31/11
     atg aaa tgc agc tgg ggc atg ttc ttc ctg atg gca gtg gtt aca ggg gtc aat tca GAG
     M   K   C   S   W   G   M   F   F   L   M   A   V   V   T   G   V   N   S   E

     61/21                                     91/31
     GTT CAG CTG CAG CAG TCT GGG GCT GAG CTT GTG AGG CCA GGG GCC TTA GTC AAG TTG TCC
     V   Q   L   Q   Q   S   G   A   E   L   V   R   P   G   A   L   V   K   L   S

                             CDR1
     121/41                                    151/51
     TGC AAA GCT TCT GGC TTC AAC ATT AAA GAC AAC TAT ATT CAC TGG GTG AGG CAG AGG CCT
     C   K   A   S   G   F   N   I   K   D   N   Y   I   H   W   V   R   Q   R   P

                                 CDR2
     181/61                                    211/71
     GAA CAG GGC CTG GAG TGG ATT GGA AGG ATT GAT CCT GAG AAT GGT TAT AGT ATA TAT GAC
     E   Q   G   L   E   W   I   G   R   I   D   P   E   N   G   Y   S   I   Y   D

     241/81                                    271/91
     CCG AAG TTC CAG GGC AAG GCC AGT ATA ACA GCA GAC ACA TCC TCC AAC ACA GCC TAC CTG
     P   K   F   Q   G   K   A   S   I   T   A   D   T   S   S   N   T   A   Y   L

     301/101                                   331/111
     CAG TTC AGC AGC CTG ACA TCT GAG GAC ACT GCC GTC TAT TAC TGT GCT AAG GAT GAT TAC
     Q   F   S   S   L   T   S   E   D   T   A   V   Y   Y   C   A   K   D   D   Y

                 CDR3
     361/121                                   391/131
     TAC CGG GTA GGG TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT
     Y   R   V   G   F   A   Y   W   G   Q   G   T   L   V   T   V   S

(B) S2C11Vн
     1/1                                       31/11
     atg gaa tgg agc tgg atc ttt atc ttt ttg gta gca aca gct aca ggt gtc cac tcc CAG
     M   E   W   S   W   I   F   I   F   L   V   A   T   A   T   G   V   H   S   Q

     61/21                                     91/31
     GTC CAG GTG AAG CAG TCT GGG GCT GAG CTG GTG AGG CCT GGG GCT TCA GTG AAG ATG TCC
     V   Q   V   K   Q   S   G   A   E   L   V   R   P   G   A   S   V   K   M   S

                             CDR1
     121/41                                    151/51
     TGC AAG GCT TCT GGC TAC ATT TTC ACC AGC TAC TTG CTG GAC TGG ATA AAA GAG AGG CCT
     C   K   A   S   G   Y   I   F   T   S   Y   L   L   D   W   I   K   E   R   P

                                 CDR2
     181/61                                    211/71
     GGA CAA GGC TTT GAG TGG ATT GGA AAT ATT AAT CCT AAT AGT GGT AGT ACT GAC TAC AAT
     G   Q   G   F   E   W   I   G   N   I   N   P   N   S   G   S   T   D   Y   N

     241/81                                    271/91
     GAA AAG TTC AAG AAT AAG GCC ACA CTG ACT GTA GAC ACA TCC TCC AGT ACA GTC TAC ATG
     E   K   F   K   N   K   A   T   L   T   V   D   T   S   S   S   T   V   Y   M

     301/101                                   331/111
     CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT GCA ATG TCC TTC ATT
     Q   L   S   S   L   T   S   E   D   S   A   V   Y   Y   C   A   M   S   F   I

             CDR3
     361/121                                   391/131
     TAT TAC TCC GAT GGT AGA TAC CGT TTT GCT TAC TGG GGC CAG GGG ACT CTG GTC AGT GTC
     Y   Y   S   D   G   R   Y   R   F   A   Y   W   G   Q   G   T   L   V   S   V

     421/141
     TCA
     S
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Figure 5. The Vн gene sequences of S2E1 (A) and S2C11 (B). CDRs are underlined. Italic letters and small letters indicate the primer and the leader sequence, respectively. The sequences are available from the GenBank database under Accession Nos AF 140611 (A) and AF 140612 (B).

5. The variable region gene sequence for light chain of S2E1 was aligned with that of S2C11. Vκ genes of both S2E1 and S2C11 are encoded by identical members of the Vκ4/5 gene family. S2C11 utilzes the Jκ4 gene segment without nucleotide change but S2E1 utilizes Jκ5

gene segment with a nucleotide change. Overall light chain variable region homology between these two Abs at the derived amino acid sequence level is 83% (14 substitutions in 107 amino acids).

V_H gene of S2C11 is encoded by a member of the V_H1 gene family, while V_H gene of S2E1 is encoded by a member of V_H14 gene family. S2C11 utilizes the DFL16.1 segment with four nucleotide changes and J_H3 gene segment also with four nucleotide changes. S2E1 appears to utilize the DSP2.9 gene segment with one nucleotide mismatch. In addition, S2E1 expresses the J_H3 gene segment.

DISCUSSION

We generated and characterized two monoclonal Abs, S2E1 and S2C11, which were able to react with HBsAg derived from HBV-infected human plasma as well as with HBsAg expressed by the liver tissue. S2E1 also bound to denatured forms of the small HBsAg, but S2C11 did not. This finding suggest that the epitope recognized by S2E1 might be linear and the epitope recognized by S2C11 might be conformation-dependent.

The sequence analyses encoding the variable regions of S2C11 and S2E1 indicate that the usage of $V\kappa$ gene by both S2E1 and S2C11 is identical as members of the $V\kappa4/5$ gene family. In spite of the different usage of $J\kappa$ gene segment by these two Abs, overall homology of variable region of κ chain between these two Abs at the derived amino acid sequence level is 83% and homology of CDRs reaches to 88.5%.

Three monoclonal anti-HBs Abs named A1.2, A2.1 and A3.1, was reported to utilize a member of $V\kappa21$ gene family and $J\kappa4$ gene family[19]. Although the usage of $V\kappa$ gene by A1.2 is different from S2E1 and S2C11, light chain variable region of A1.2 showed 83% homology with S2C11 and 79.5% homology with S2E1.

V_H gene of S2C11 is encoded by a member of the V_H1 gene family which has the largest number of members[20], while V_H gene of S2E1 is encoded by a member of V_H14 gene family. Both S2C11 and S2E1 express J_H3 gene segment but S2C11 and S2E1 express different D gene segment (DFL16.1 gene segment and the DSP2.9 gene segment, respectively). Homology of heavy chain variable regions of these Abs at the amino acid sequence level with monoclonal anti-HBs Abs reported by others was not significant[19,21,22].

Considering the expression of closely related light chains by both S2C11 and S2E1 and the inability of S2C11 to recognize the denatured form of the small HBsAg, it is likely that their epitope specificity is attributed to their heavy chains. This is further supported by the fact that there is high percent residue identities of light chain variable regions of these Abs with A1.2 monoclonal anti-HBs Abs reported by KL Lohman who suggested its epitope fine specificity was determined by its heavy chains. In addition, there is a report that only Fd of heavy chain was sufficient for the binding of a human monoclonal Ab (B7) to HBsAg[23]. We are under the work to express $V\kappa$ and V_H of both S2C11 and S2E1 and to determine the involvement of $V\kappa$ of these Abs in binding to HBsAg.

Further, cDNA of these Abs will be used in constructing eukaryotic expression vector of single chain Fv in order to express intracellular Ab which can inhibit HBsAg production and HBV replication.

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