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ACCEPTED

1 **Generation and characterization of a neutralizing human monoclonal antibody to**
2 **hepatitis B virus preS1 from phage-displayed human synthetic Fab library**

3

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17

18 **Running title: An anti-hepatitis B virus preS1 neutralizing human monoclonal antibody**

19 **Abstract**

20 The hepatitis B virus (HBV) envelope contains small (S), middle (M), and large (L) proteins.

21 PreS1 of the L protein contains a receptor-binding motif crucial for HBV infection. This

22 motif is highly conserved among 10 HBV genotypes (A–J), making it a potential target for

23 the prevention of HBV infection. In this study, we successfully generated a neutralizing

24 human monoclonal antibody (mAb), 1A8 (IgG1), that recognizes the receptor-binding motif

25 of preS1 using a phage-displayed human synthetic Fab library. Analysis of the antigen-

26 binding activity of 1A8 for different genotypes indicates that it can specifically bind to the

27 preS1 of **major** HBV genotypes (**A–D**). Based on Bio-Layer interferometry, the affinity (K_D)

28 of 1A8 for the preS1 of genotype C was 3.55 nM. 1A8 immunoprecipitated the HBV virions

29 of genotypes C and D. In an *in vitro* neutralization assay using HepG2 cells overexpressing

30 the cellular receptor sodium taurocholate cotransporting polypeptide, 1A8 effectively

31 neutralized HBV infection with genotype D. Taken together, the results suggest that 1A8 may

32 neutralize the **four** HBV genotypes. Considering that genotypes A–D are most prevalent, 1A8

33 may be a neutralizing human mAb with promising potential in the prevention and treatment

34 of HBV infection.

35

36 **Keywords**

37 Hepatitis B virus, PreS1, Human monoclonal antibody, Phage display, Synthetic antibody

38 library, Neutralizing antibody.

39

40 **Introduction**

41 Hepatitis B virus (HBV) infection is one of the most serious and prevalent public health
42 problems. Approximately 257 million people are chronically infected with HBV, resulting in
43 887,000 deaths in 2015, mostly from complications of acute or chronic HBV infection,
44 including liver cirrhosis and hepatocellular carcinoma [1]. To date, 10 distinct HBV
45 genotypes (A–J) with different geographical distributions have been identified. Genotypes A
46 and D are widespread in Africa and Europe; genotypes B and C are prevalent in Asia; and
47 genotypes E–J are occasionally encountered in Europe, America, and Asia. Genotypes A–D
48 are most prevalent and responsible for approximately 90% of hepatitis B globally [2].

49 The HBV envelope contains three structurally related small (S), middle (M), and large (L)
50 proteins. The S protein is the common C-terminal domain of these envelope proteins, the M
51 protein consists of preS2 and S, and the L protein consists of preS1, preS2, and S [3]. In
52 addition to virions, HBV-infected hepatocytes also produce non-infectious spherical and
53 filamentous subviral particles. These subviral particles mainly consist of S proteins and
54 typically outnumber virions by 1,000 to 10,000:1. Thus, these particles have been suspected
55 to reduce the virus-specific immune response by mimicking virions [4]. Unlike S protein, the
56 preS1 of L protein exists primarily in infectious virions. In addition, amino acids 20–26 of
57 preS1 play an essential role in the interaction with the cellular receptor sodium taurocholate
58 cotransporting polypeptide (NTCP) and mediate HBV infection [5, 6]. This essential region is
59 highly conserved among HBV genotypes (Fig. 1), making it a potential target for the
60 prevention and treatment of HBV infection.

61 Hepatitis B immunoglobulin (HBIG) is currently used as a post-exposure prophylactic
62 medical treatment for accidental or perinatal HBV exposure. HBIG is prepared by collecting
63 the serum of high anti-S protein antibody titers. Considering that the preS1 region is crucial

for HBV infection, preS1-specific monoclonal antibody (mAb) may represent a promising approach for the prevention and treatment of HBV infection. Clinically, the appearance of anti-preS1 antibodies in patients correlates well with better recovery from acute hepatitis B [7]. To date, several anti-preS1 mAbs with HBV-neutralizing activity *in vivo* or *in vitro* have been generated by immunizing mice with HBV particles or recombinant preS1 antigen [8-14]. However, most of the antibodies do not neutralize all of the HBV genotypes. Therefore, we aimed to generate mAbs against the highly conserved essential region of preS1 for more effective prevention strategies. In the present study, using a phage-displayed human synthetic Fab library, we describe the successful generation of a human mAb, 1A8, which binds to the highly conserved receptor-binding motif of preS1 and neutralizes HBV infection *in vitro*.

74

75 **Materials and methods**

76 **Cell culture**

77 Suspension-adapted HEK293F cells (Invitrogen) were grown in FreeStyle 293 Expression
78 medium (Gibco) at 125 rpm, 37°C in a humidified incubator with 8% CO₂. Human hepatoma
79 HepG2 cells were grown in DMEM (Gibco), respectively, supplemented with 10% fetal
80 bovine serum (FBS; GE Healthcare Life Sciences) at 37°C in a humidified incubator with 5%
81 CO₂.

82

83 **Preparation of preS1 antigens**

84 The GST-preS1(1–119), GST-preS1(1–56), and preS1(1–119) of HBV genotype C were
85 prepared as described previously [15]. The recombinant preS1 of HBV genotypes A–G,
86 comprising the Ig1–5 domains of human L1 cell adhesion molecule (L1CAM), preS1(1–60),

87 and Strep tag, was transiently expressed in HEK293F cells for 7 days and purified by affinity
88 chromatography using a Streptactin Superflow high capacity column (Iba, Germany) as
89 described previously [9]. Biotinylated synthetic preS1 peptide (biotin–
90 SGSGNPLGFFPDHQLDP, Bio-preS1-L peptide) containing the NTCP-binding motif (20–
91 NPLGFFP–26) with >98% of purity was purchased from AnyGen, Inc. (South Korea). The
92 recombinant preS1 antigens and Bio-preS1-L peptide were used as antigens for biopanning
93 and enzyme-linked immunosorbent assay (ELISA).

94

95 **Selection of preS1-specific Fab from a phage-displayed human synthetic Fab library**

96 A human synthetic Fab library (1.35×10^9 diversity, unpublished) constructed in our
97 laboratory was panned against four preS1 antigens, GST-preS1(1–119), GST-preS1(1–56),
98 Bio-preS1-L peptide, and preS1(1–119), for the first, second, third, and fourth rounds,
99 respectively, using the standard panning procedure [16]. Briefly, an immunotube (or
100 streptavidin-coated plates in the case of Bio-preS1-L peptide) was coated with each preS1
101 antigen at 4°C overnight. Antibody library phages were incubated with the antigen and
102 unbound phages washed out with 0.1% PBST (0.1% Tween 20 in PBS). The bound phages
103 were eluted using 10 µg/ml trypsin solution at 37°C for 30 min. *Escherichia coli* TG1 cells
104 (OD₆₀₀ ~0.5) were infected with the eluted phages, grown for 1 h at 37°C, plated on a 2 ×
105 YT/carbenicillin/glucose agar plate, and incubated overnight at 37°C. The amplified phages
106 were subjected to the next round of panning, with washing stringency increased gradually
107 each round (Fig. 2A). For titration, the number of input and output phages for each panning
108 round was calculated by dilutions and colony forming units. After the four rounds of panning,
109 the output cells were grown and infected with helper phages to obtain polyclonal Fab phages.
110 These phages were subjected to indirect ELISA to confirm the enrichment of positive clones.

111 After the fourth round of panning, a total of 188 colonies were randomly selected and
112 individually grown as monoclonal Fab phages, and then subjected to indirect and quantitative
113 ELISAs. For indirect ELISA, 100 ng of BSA, Bio-preS1-L peptide, preS1(1–119), or
114 recombinant preS1 was used as an immobilized antigen and horseradish peroxidase (HRP)-
115 conjugated anti-M13 (1:5000 v/v, GE Healthcare) as a secondary antibody. For quantitative
116 ELISA, anti-M13 antibody (100 ng/well, GE Healthcare) and anti-human kappa-HRP
117 (1:2000 v/v, Novex) were used as the immobilized antigen and secondary antibody,
118 respectively, as described previously [17].

119

120 **Conversion of Fab into IgG1 and expression in HEK293F cells**

121 To convert the selected Fab into IgG1 format, the heavy chain variable region (VH) and
122 kappa light chain variable region (VK) sequences were amplified by PCR and combined with
123 the IgG heavy and light chain leader sequences, respectively, using recombinant PCR. The
124 resulting VH and VK sequences were subcloned into the *EcoRI-ApaI* and *HindIII-BsiWI* sites
125 of the pdCMV-*dhfC* expression plasmid containing the human C γ 1 and C κ genes,
126 respectively. The resulting expression plasmids were introduced into HEK293F cells using
127 polyethyleneimine (PEI, linear 25 kDa; Polysciences, USA) at a ratio of 1:4 (60 μ g:240 μ g)
128 as described previously [18]. The transfected cells were cultured for 7 days, the culture
129 supernatants filtered using a bottle top filter (0.22 μ m PES, Sartorius) for antibody
130 purification.

131

132 **Purification of anti-preS1 human mAb**

133 The culture supernatants were subjected to affinity chromatography on Protein A-agarose

134 beads (Amicogen, Korea) as described previously [9]. The antibody concentration was
135 determined by a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). The
136 integrity and purity of the purified antibody were assessed by sodium dodecyl sulfate
137 polyacrylamide gel electrophoresis (SDS-PAGE).

138

139 **Analysis of antigen-binding activities of purified 1A8 mAb**

140 To analyze the antigen-binding activities of purified 1A8 mAb to the preS1 antigens of
141 different HBV genotypes, the recombinant preS1 antigen (200 ng) of different genotype was
142 individually incubated with anti-L1CAM antibody A10-A3 (200 ng) coated on each well,
143 then bound preS1 antigen was incubated with serially diluted 1A8 antibody. The bound 1A8
144 antibody was detected using anti-human IgG Fc-HRP (1:10,000 v/v, Jackson) as the
145 secondary antibody. To compare the antigen-binding activity between 1A8 and HzKR127-3.2,
146 the antibody was serially diluted and incubated with recombinant preS1 (genotype C, 100 ng)
147 coated on each well, then the bound antibody was detected using anti-human IgG Fc-HRP
148 (1:8000 v/v, Invitrogen) as the secondary antibody.

149

150 **Affinity determination**

151 For affinity determination of the antibody by Bio-Layer Interferometry (BLI) using Octet
152 RED (ForteBio), anti-human Fc-coated biosensor tips (AHC, ForteBio) were activated in
153 0.1% PBA (0.1% BSA in PBS) for 20 min by agitating a 96-well microtiter plate (Greiner
154 Bio-One) at 1000 rpm. Antibody (1 µg/ml, 200 µl) was captured for 10 min and washed with
155 0.1% PBA for 3 min. Purified recombinant preS1 of HBV genotype A was prepared as a 2-
156 fold serial dilution (100, 50, 25, 12.5, and 6.25 nM) with 0.1% PBA and separately incubated

157 with antibody bound on the tips. Association and dissociation rates were measured for 10 and
158 20 min, respectively. All measurements were corrected for baseline drift by subtracting a
159 control sensor (antibody-captured AHC sensor) exposed to running buffer only. The operating
160 temperature was maintained at 30°C. Data were analyzed using a 1:1 interaction model
161 (fitting global, Rmax unlinked by sensor) in ForteBio data analysis software version 7.1.

162

163 **Western blot analysis**

164 GST-preS1(1–56) was expressed in *E. coli* DH5a with 0.2 mM isopropyl-BD-
165 thiogalactopyranoside (IPTG) for 2 h, as described previously [19]. Cell lysates from
166 uninduced or IPTG-induced cells and 10 µg of purified GST-preS1(1–56) were separated by
167 12% SDS-PAGE, transferred to nitrocellulose membrane, and incubated with antibody (0.5
168 µg/ml, 10 ml), followed by anti-human IgG Fc-HRP conjugate (1:6000 v/v, Thermo
169 Scientific). The bands were visualized using a chemiluminescent substrate (WEST-ZOL plus,
170 iNtRON BioTechnology, Korea).

171

172 **Immunoprecipitation assay**

173 To produce HBV particles, HepG2 cells were seeded at a density of 6×10^5 cells in 6-well
174 plates and cultured at 37°C. The next day, the HepG2 cells were transfected with pHBV5.2
175 (HBV genotype C) or pHBV1.2 (HBV genotype D) using Lipofectamine 2000 (Invitrogen)
176 [20, 21], and then incubated for 4 days. The culture supernatants were harvested and
177 incubated with 1 µg/ml antibody overnight at 4°C, and then immunoprecipitated with 20 µl of
178 protein A beads for 6 h at 4°C. After washing the immunoprecipitated complex three times
179 with PBS, the viral DNA was detected as described previously [20]. Briefly, the

180 immunocomplex was treated with DNase I (Sigma) and mung bean nuclease (Takara,
181 Kusatsu, Shiga, Japan) at 37°C for 20 min to remove transfected plasmid DNA. The core-
182 associated HBV DNA was prepared by digestion with Proteinase K (20 mg/ml, Roche) at
183 37°C for 2–3 h in the presence of 0.5% SDS. HBV DNA was extracted using
184 phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma) and precipitated with ethanol and 3 M
185 sodium acetate. Purified DNA was separated on a 1% agarose gel and HBV DNA was
186 detected by Southern blot hybridization using ³²P-labeled HBV probe [20, 22].

187

188 ***In vitro* HBV-neutralization assay**

189 The HBV-neutralizing activity of 1A8 was investigated using a HepG2-NTCP stable cell
190 line. The HepG2-NTCP cells were seeded at a density of 6×10^5 cells in 6-well plates and
191 cultured at 37°C. The next day, the HepG2-NTCP cells were infected with HBV particles of
192 genotype D (~2000 viral genome equivalents per cell) in primary hepatocyte maintenance
193 medium (PMM) containing 4% PEG and 2.5% DMSO as described previously [20]. For the
194 neutralization assay, the HBV particles were pre-incubated with each antibody at different
195 concentrations for 1 h at room temperature, and then added to the cultured HepG2-NTCP
196 cells. The medium was changed every 2 days for PMM supplemented with 2.5% DMSO and
197 the infected cells harvested 7 days post-infection. The intracellular HBV DNA was extracted
198 from the infected HepG2-NTCP cells and subjected to Southern blot hybridization.

199

200 **Results**

201 **Isolation of Fabs specific to HBV preS1 from a phage antibody library**

202 To isolate Fabs specific to preS1 of HBV, a phage-displayed human synthetic Fab library

203 (1.35 × 10⁹ diversity, unpublished) was panned against four preS1 antigens: GST-preS1(1–
204 119), GST-preS1(1–56), Bio-preS1-L peptide, and preS1(1–119) [15]. The output phage titers
205 increased after the third round of panning against the Bio-preS1-L peptide (Fig. 2A), and the
206 antigen-binding activity of polyclonal output phages to preS1 antigens increased significantly,
207 as assessed by indirect ELISA (Fig. 2B). After the fourth round of panning, 188 phage Fab
208 clones were randomly selected and their antigen-binding activities analyzed by indirect
209 ELISA using preS1(1–119), Bio-preS1-L peptide, and recombinant preS1 of genotypes A and
210 D. The 1A8 phage Fab had the highest antigen-binding activity with preS1 and was selected
211 for further study (Fig. 2C).

212

213 **Conversion of 1A8 Fab into human IgG1 and analysis of antigen-binding activity**

214 The 1A8 Fab was converted to human IgG1 and transiently expressed in HEK293F cells,
215 and the culture supernatants subjected to protein purification. The integrity and purity of
216 purified 1A8 IgG1 were confirmed by SDS-PAGE (Fig. 3A). The purified 1A8 bound to the
217 recombinant preS1 of genotypes A–D; its antigen-binding activity to genotype A–C was the
218 same and approximately 35% higher than to genotype D (Fig. 3B). However, 1A8 hardly
219 bound to preS1 of genotype E, and did not bind to genotype F and G. In addition, the antigen-
220 binding activity of 1A8 to preS1(1–119) of genotype C was slightly lower than that of
221 HzKR127-3.2 [8], a humanized anti-preS1 mAb with a K_D of 0.5 nM (Fig. 3C). Affinity
222 determination by BLI using Octet Red indicated that the K_D of 1A8 for the recombinant
223 preS1 of genotype A was approximately 3.55 nM (Fig. 3D).

224

225 **Validation of the antigen-binding specificity of 1A8**

226 To evaluate the antigen-binding specificity of 1A8, GST-preS1(1–56) was expressed in *E.*
227 *coli* DH5a and the lysates of uninduced or induced cells subjected to Western blot analysis for
228 1A8 with purified GST-preS1(1–56) as a positive control. As shown in Fig. 4, 1A8 exhibited
229 antigen-binding specificity to preS1 without any non-specific binding activity to endogenous
230 protein of *E. coli*.

231

232 **Evaluation of the HBV-neutralizing activity of 1A8**

233 Prior to assessing the *in vitro* HBV-neutralizing activity of 1A8, an immunoprecipitation
234 assay was performed to determine whether 1A8 can bind the HBV virion. Previously
235 developed HBV-neutralizing humanized anti-preS1 mAb HzKR127-3.2 was used as a
236 positive control for HBV genotypes C and D, whereas humanized anti-preS1 mAb
237 HzKR359-1 was used as a positive control for genotype C and a negative control for
238 genotype D [8, 9]. Mouse IgG was used as a negative control. Viral DNA was extracted from
239 immunoprecipitates and measured by Southern blot hybridization. As shown in Fig. 5A, the
240 relaxed circular (RC) and double-stranded linear (DSL) forms of HBV DNA were detected in
241 1A8 precipitates, indicating that 1A8 could bind to both HBV genotypes C and D.

242 To examine whether 1A8 can neutralize HBV infection, HBV particles of genotype D were
243 pre-incubated with each antibody at different concentrations, and then added to cultured
244 HepG2-NTCP cells overexpressing cellular receptor NTCP. Seven days post-infection, the
245 infected cells were harvested and the intracellular HBV DNA measured by Southern blot
246 hybridization. Although 1A8 had lower binding activity to preS1 of genotype D compared to
247 genotype C (Fig. 3B), it effectively neutralized infection with both HBV genotypes in a dose-
248 dependent manner (Fig. 5B). The neutralizing efficacy was similar to that of HzKR127-3.2,
249 which has a higher affinity than 1A8. This result is consistent with the immunoprecipitation

250 assay.

251

252 **Discussion**

253 PreS1 of HBV contains a highly conserved receptor-binding motif that plays an essential
254 role in HBV infection and mediates HBV infection. However, HBIG, which is currently used
255 as a post-exposure prophylactic medical treatment, contains mostly anti-S protein antibodies.
256 The subviral particles are usually present in 10,000-fold excess over complete HBV particles
257 in the blood of infected persons. Therefore, mAbs targeting the receptor-binding motif of
258 preS1 may represent a promising approach for effective prevention and treatment of HBV
259 infection. In this study, we successfully generated a broadly neutralizing human mAb specific
260 to the receptor-binding motif by panning a phage-displayed human synthetic Fab library
261 against preS1 antigens comprising the short peptide containing the receptor-binding motif.
262 Analysis of the antigen-binding activity indicated that 1A8 can bind to the preS1 of major
263 four HBV genotypes (A–D). In addition, 1A8 bound the HBV particles of both genotypes C
264 and D and effectively neutralized infection with HBV of genotype D *in vitro*. The results
265 suggest that 1A8 may neutralize HBV genotypes A–D. Given that these genotypes are
266 responsible for approximately 90% of hepatitis B globally [2], 1A8 may represent a
267 neutralizing human mAb that can be used for the prevention and treatment of HBV infection.

268 Regarding the epitope of 1A8, it exhibited reduced binding activity towards preS1 of
269 genotypes D or E compared to genotypes A–C, indicating that the amino acid at position 19
270 influences the antigen-binding activity of 1A8, and that Pro19 is optimal for antigen binding.
271 In addition, 1A8 did not exhibit antigen-binding activity toward preS1 of genotypes F or G,
272 indicating that Phe25 is essential for antibody binding. The results suggest that 1A8
273 recognizes aa 20–25 in the receptor-binding motif and, thus, neutralize HBV infection by

274 blocking the preS1-NTCP interaction.

275 To date, several murine anti-preS1 mAbs with HBV-neutralizing activity *in vivo* or *in vitro*
276 have been reported, but these antibodies exhibit limited neutralizing activities against some
277 HBV genotypes, in addition to the immunogenicity issue [12-14]. In our previous study, we
278 developed HBV-neutralizing humanized anti-preS1 mAb HzKR127-3.2 and HzKR359-1 [8,
279 9]. HzKR127-3.2 exhibited binding activity only to preS1 of genotypes A, C, D, and G,
280 whereas HzKR359-1 bound to genotypes A, B, and C. To our knowledge, 1A8 is the first
281 human anti-preS1 mAb that may neutralize major four HBV genotypes (A-D). Considering
282 that the presence of anti-preS1 antibodies in patients correlates with better recovery from
283 acute hepatitis B, and that antibody-mediated immunotherapy could be effective for chronic
284 hepatitis B [7, 23], 1A8 may be effective in the prevention of HBV infection and therapy for
285 hepatitis B.

286

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290

291 **Conflict of interest**

292 We have no conflict of interest to declare.

293

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358

359

360 **Figure legends**

361 **Figure 1. Construction of recombinant preS1 antigens of different HBV genotypes.**

362 Amino acid sequence alignment of the HBV preS1 N-terminal receptor-binding region
363 (genotypes A–J, numbering based on HBV genotype A). The essential receptor-binding motif
364 of preS1 (amino acids 20–26) is shaded in gray. The sequence contained in the [Bio-preS1-L](#)
365 [peptide](#) is underlined.

366

367 **Figure 2. Isolation of anti-preS1 monoclonal Fab from a human synthetic Fab library.**

368 A) Input and output phage titers of each panning round. [pfu, plaque-forming unit.](#) B)
369 Enrichment of positive polyclonal phage clones as determined by indirect ELISA. The fourth
370 round of panning with maximal enrichment against preS1 was chosen for the isolation of
371 monoclonal Fab. Values were obtained from duplicate wells and are expressed as mean ±
372 SEM. C) The antigen-binding activity of 1A8 phage Fab was assessed by indirect ELISA
373 using the preS1(1–119) of [genotype C](#), Bio-preS1-L peptide, or recombinant preS1 of HBV
374 genotypes A or D.

375

376 **Figure 3. Analysis of the antigen-binding activity of purified 1A8 mAb.**

377 A) [SDS-PAGE](#) of purified 1A8 under non-reducing (NR, 6%) and reducing (R, 10%)
378 conditions. M, protein marker; HC, heavy chain; LC, light chain. B) The antigen-binding
379 activities of purified 1A8 mAb to the preS1 of different HBV genotypes (A–G) were assessed
380 by ELISA. C) The antigen-binding activity of purified 1A8 was compared to humanized anti-
381 preS1 mAb HzKR127-3.2 by indirect ELISA using the recombinant preS1 of [HBV genotype](#)

382 C. D) Affinity determination of 1A8 by BLI using Octet RED. The recombinant preS1 of
383 HBV genotype A was prepared as a 2-fold serial dilution (100, 50, 25, 12.5, and 6.25 nM).
384 All values were obtained from duplicate wells and are expressed as mean ± SEM.

385

386 **Figure 4. Western blot analysis of 1A8 mAb**

387 A) GST-preS1(1–56) was expressed in *E. coli* DH5a and subjected to 12% SDS-PAGE (left)
388 followed by Western blot analysis with 1A8 (right). M, protein marker; lane 1, uninduced cell
389 lysates; lane 2, IPTG-induced cell lysates; lane 3, **purified** GST-preS1(1–56) with the
390 molecular weight of 34 kDa.

391

392 **Figure 5. Evaluation of the virion-binding activity and *in vitro* HBV-neutralizing activity
393 of 1A8 against HBV of genotype D.**

394 A) Immunoprecipitation of HBV particles (genotypes C and D) by 1A8. HzKR127-3.2 was
395 used as a positive control for **genotypes C and D**, whereas HzKR359-1 was used as a positive
396 control for genotype C and a negative control for genotype D. Mouse IgG was used as a
397 negative control for both genotypes. RC DNA, relaxed circular DNA; DSL DNA, double-
398 stranded linear DNA. B) HBV particles (**genotype D**) were pre-incubated with 1A8 or
399 HzKR127-3.2 (10, 1, 0.1 µg), and then added to cultured HepG2-NTCP cells. The medium
400 was changed every 2 days and infected cells harvested 7 days post-infection. The intracellular
401 HBV DNA was extracted and subjected to Southern blot hybridization.

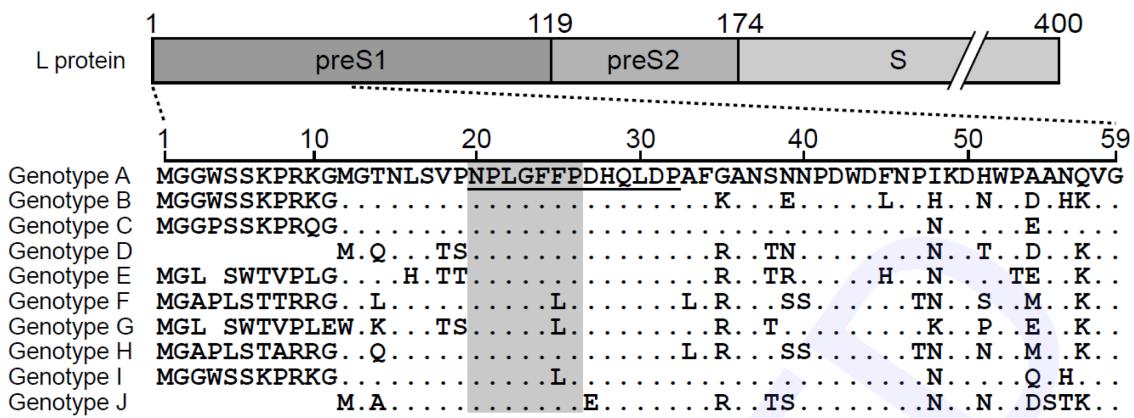


Fig. 1. Figure 1. Construction of recombinant preS1 antigens of different HBV genotypes.

Amino acid sequence alignment of the HBV preS1 N-terminal receptor-binding region (genotypes A-J; numbering based on HBV genotype A). The essential receptor-binding motif of preS1 (amino acids 20-26) is shaded in gray. The sequence contained in the Bio-preS1-L peptide is underlined.

A

Round	Antigen	Wash	Input phage titer (pfu/ml)	Output phage titer (pfu/ml)
1R	GST-preS1 (1-119)	10	1.62×10^{14}	1.42×10^6
2R	GST-preS1 (1-56)	13	1.76×10^{14}	1.0×10^6
3R	PreS1 peptide	15	3.10×10^{13}	6.33×10^7
4R	PreS1 (1-119)	20	6.90×10^{13}	5.49×10^9

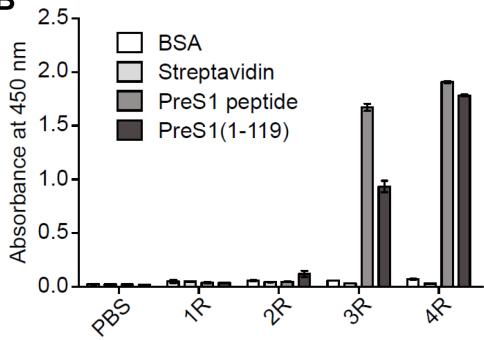
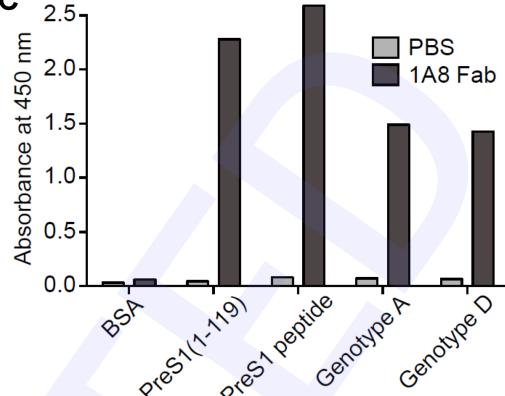
B**C**

Fig. 2. Figure 2. Isolation of anti-preS1 monoclonal Fab from a human synthetic Fab library.

A) Input and output phage titers of each panning round. pfu, plaque-forming unit. B) Enrichment of positive polyclonal phage clones as determined by indirect ELISA. The fourth round of panning with maximal enrichment against preS1 was chosen for the isolation of monoclonal Fab. Values were obtained from duplicate wells and are expressed as mean

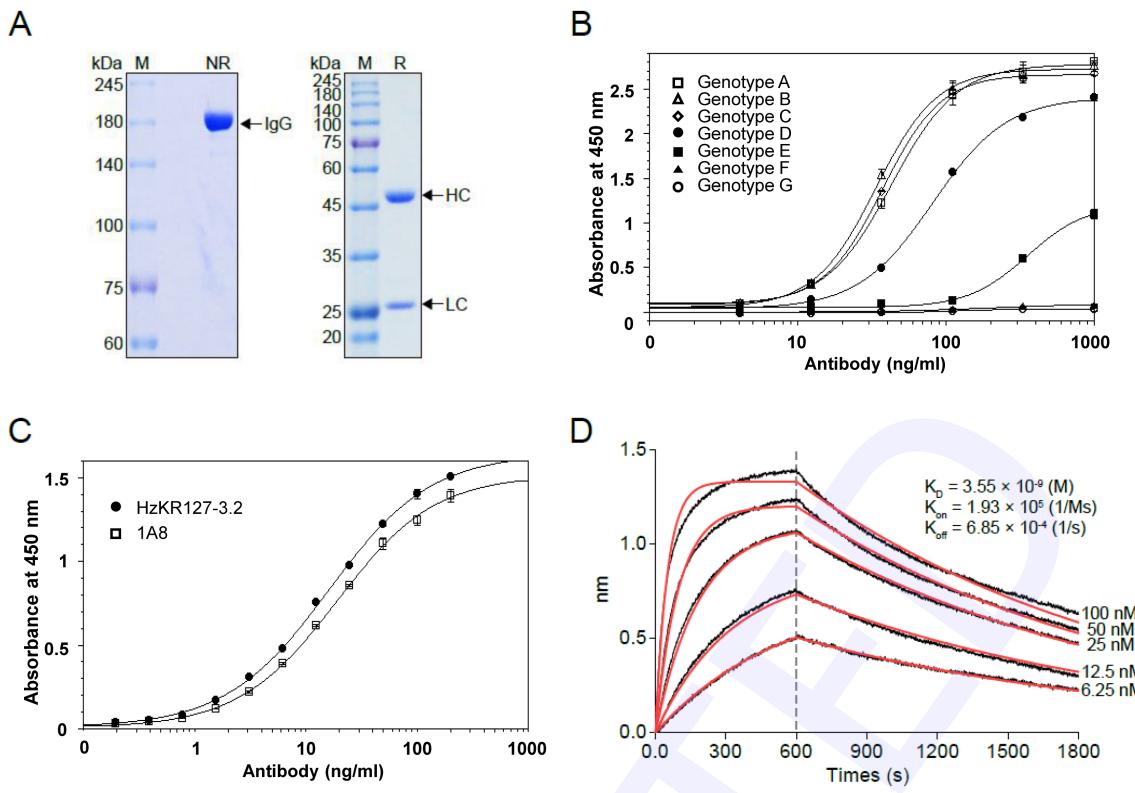


Fig. 3. Figure 3. Analysis of the antigen-binding activity of purified 1A8 mAb.

A) SDS-PAGE of purified 1A8 under non-reducing (NR, 6%) and reducing (R, 10%) conditions. M, protein marker; HC, heavy chain; LC, light chain. B) The antigen-binding activities of purified 1A8 mAb to the preS1 of different HBV genotypes (A-G) were assessed by ELISA. C) The antigen-binding activity of purified 1A8 was compared to humanized anti-preS1 mAb HzKR127-3.2 by indirect ELISA using the recombinant preS1 of HBV genotype C. D) Affinity determination of 1A8 by BLI using Octet RED. The recombinant preS1 of HBV genotype A was prepared as a 2-fold serial dilution (100, 50, 25, 12.5, and 6.25 nM). All values were obtained from duplicate wells and are expressed as mean

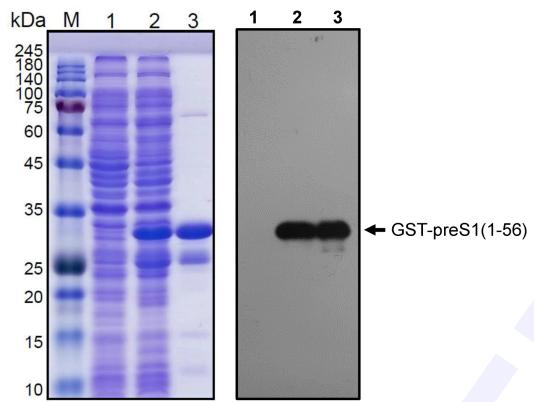


Fig. 4. Figure 4. Western blot analysis of 1A8 mAb

A) GST-preS1(1–56) was expressed in *E. coli* DH5 α and subjected to 12% SDS-PAGE (left) followed by Western blot analysis with 1A8 (right). M, protein marker; lane 1, uninduced cell lysates; lane 2, IPTG-induced cell lysates; lane 3, purified GST-preS1(1–56) with the molecular weight of 34 kDa.

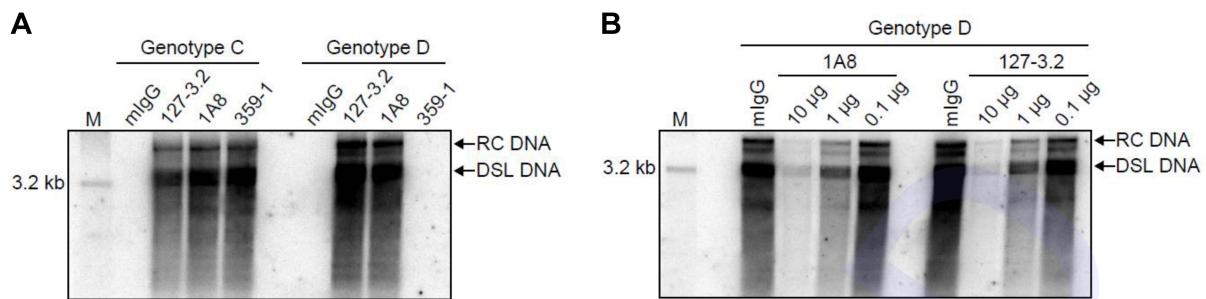


Fig. 5. Figure 5. Evaluation of the virion-binding activity and in vitro HBV-neutralizing activity of 1A8 against HBV of genotype D.

A) Immunoprecipitation of HBV particles (genotypes C and D) by 1A8. HzKR127-3.2 was used as a positive control for genotypes C and D, whereas HzKR359-1 was used as a positive control for genotype C and a negative control for genotype D. Mouse IgG was used as a negative control for both genotypes. RC DNA, relaxed circular DNA; DSL DNA, double-stranded linear DNA. B) HBV particles (genotype D) were pre-incubated with 1A8 or HzKR127-3.2 (10, 1, 0.1