Synthesis of Human Antibodies Against HBsAg in Newly Established Chinese Hamster Lung (CHL-YN) Cell Line

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Abstract: Hepatitis B immunoglobulin (HBIG) is an effective treatment for hepatitis B, including postexposure prophylaxis of HBV infection, prevention of HBV reinfection in liver transplant patients, and reducing sexual transmission. This study investigated the synthesis of human IgG antibodies that specifically target HBsAg subtype *adr* in CHL-YN cells, a newly established cell line that grows faster than CHO-K1. To achieve the synthesis of human IgG antibodies, a plasmid vector encoding DNA sequences for human IgG antibodies against HBsAg was constructed and then transiently transfected into CHL-YN cells. The expression and antigen-binding capacity of the recombinant human IgG antibodies were analyzed using western blot and ELISA. The results showed successful expression and secretion of human IgG antibodies that recognize HBsAg subtype *adr* in CHL-YN cells. The ELISA test confirmed the specificity of the human IgG antibodies towards HBsAg subtype *adr*. Thus, this study concluded that human IgG antibodies that target HBsAg subtype *adr* were transiently expressed in CHL-YN cells.

Keywords: *antibodies; hepatitis B; CHL-YN cells; transient expression; vector plasmid.*

1 Introduction

Hepatitis B is a serious disease caused by infection with the hepatitis B virus (HBV). HBV infection can cause acute and chronic hepatitis B disease. Chronic infection with HBV can cause other serious illnesses such as cirrhosis and liver cancer. In 2015, approximately 257 million people in the world lived with chronic hepatitis B [1]. In Indonesia, approximately 28 million people are infected with

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hepatitis B and C [2]. In 2015, approximately 887,000 death cases were caused by hepatitis B (generally cirrhosis and liver cancer) in the world [1]. To prevent disease from developing due to HBV infection, prevention and treatment is very important.

One of the available treatments for hepatitis B is the use of therapeutic antibodies, called hepatitis B immunoglobulin (HBIG). After liver transplantation, hepatitis B patients are treated with HBIG every day for one week to maximize protection from reinfection in the liver graft. HBIG treatment is also used in conjunction with the hepatitis B vaccine for infants born to hepatitis B positive mothers. In addition, HBIG is also known to be effective as a treatment for hepatitis B patients who are infected through sexual contact [3]. HBIG is isolated from positive donor plasma with safety guaranteed by stringent product standards, but doing so increases production costs and prices for the consumer [4]. Therefore, it is desirable to have an alternative method to produce HBIG.

Many studies have been reported that produced recombinant human monoclonal antibodies that can recognize HBsAg antigens. Parental recombinant human monoclonal antibodies that can recognize HBsAg antigens, called CL4mAb, are produced in human TAPC301-CL4 cells with Epstein-Barr virus [5-7]. Based on previous research, it is known that these antibodies have the ability to neutralize the activity of HBV subtype *adr* in vivo, however, these monoclonal antibodies are not recommended for human use because viruses are used in their production [8]. Based on previous research, CL4mAb has also been successfully produced in *Escherichia coli*, however, the prokaryotic expression system has the disadvantage of not having the post-translational modification ability [9,10]. In addition, CL4mAb has also been successfully produced in tobacco plants, but a drawback of plant expression is that the N-glycosylation mechanism in plants is different from that in mammals [11]. CL4mAb was successfully produced in diatoms (*Phaedactylum tricornutum*) but the N-glycan pattern of the antibodies was different from that in mammals [12].

Based on these problems, mammalian cells can be an alternative for the production of CL4mAb. Mammalian cells are cells that are commonly used for production of recombinant proteins approved by the FDA. One of the mammalian cells that have potential to become host cells in the production of CL4mAb are CHL-YN cells. CHL-YN cells are newly established cells that were developed in 2018 by Noriko Yamano-Adachi. CHL-YN cells are isolated from the lung organ of the Chinese hamster (*Cricetulus griseus*). CHL-YN cells have various advantages as host cells for antibody production. For example, CHL-YN cells can modify protein post-translationally. CHL-YN cells have been reported to have a relatively fast division time (around 10.74 hours ± 0.20 hours) compared to CHO-K1 cells (21.29 ± 0.34 hours). Further, the antibodies produced with

CHL-YN cells have been reported to have a similar N-glycan pattern to the IgG1 antibodies that were produced by commonly used cell lines. CHO-K1 CHL-YN cells have also been reported to have a better ability to produce antibodies than CHO-K1 cells. The IgG1-production rate in CHL-YN cells (antibody production rate 0.2554 ± 0.0115 pg $cell^{-1}day^{-1}$) is two times faster than in CHO-K1 cells (antibody production rate 0.1239 ± 0.0232 pg $cell^{-1}day^{-1}$). Moreover, CHL-YN cells can produce IgG1 (7.13 ± 0.29 mg/L) with a higher concentration than CHO-K1 cells (6.05 ± 0.73 mg/L) [13].

This study aimed to synthesize human IgG antibodies that can recognize HBsAg subtype *adr* (CL4mAb) in CHL-YN cells. In the future, CHL-YN cells may become an alternative host cell for human IgG production.

2 Materials and Methods

2.1 Plasmid Construction

DNA sequences for light and heavy chains of monoclonal IgG against HBsAg were obtained from the GenBank Depository with accession numbers JF970210 and JF970211. Both DNA sequences were adapted to *Cricetulus griseus* specific codon usage and synthesized by Eurofins Genomics (Japan). The signal peptide in the DNA sequence was analyzed with the SignaIP 5.0 software. In this study, the amino acid sequence was analyzed for prediction of the antibody structure with the SWISS-Model software. The prediction of disulfide bonds was analyzed with the CYSPRED software. Heavy and light-chain genes were inserted into Mammalian PowerExpress System (Toyobo Co., Ltd., Japan). Heavy-chain genes were inserted into vector pEHX1.2 and light chain genes were inserted into vector pELX2.2. Both vectors were combined and a pELC2+HC vector containing heavy and light genes was generated. Endotoxins present in the plasmid were removed using the MiraCLEAN Endotoxin Removal Kit (Mirus Bio LLC, Madison, WI, USA) twice before transfection.

2.2 Transfection

CHL-YN cells were cultured in EX-CELL CD CHO fusion medium containing 6mM L-glutamine. The pELC2+HC vector containing heavy and light genes was transiently transfected by the Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Transfection was performed at 1350 V, width: 5, pulses: 10. One µg of expression vector was used for 1 x 10^6 cells per well of a six-well plate. A mock vector (not inserted with the genes) was used as negative control. For transfection control, 1 x 10^6 cells were transfected without vector. The transfected cells were incubated at 37 °C with 5% CO_2 for 72 hours.

2.3 Detection of Anti-HBsAg Antibodies

Antibodies against HBsAg were detected in the medium of transfected cells by western blot analysis under reducing condition. 48 and 72 hours after transfection, medium from the cells was isolated and centrifuged at 1000 x g for 5 minutes. 13 µL supernatant samples were loaded to SDS-PAGE. The gel was blotted on a PVDF membrane and blocked by 5% skim milk for 1 hour. The membrane was incubated overnight with secondary antibodies at 1:2000 and 4 °C. Rabbit anti-human IgG H&L conjugated with HRP was used as secondary antibody. After washing with PBST solution three times, 2 mL of substrate containing HRP substrate luminol reagent and HRP substrate peroxide solution (1:1) substrate was added to the membrane. Imaging was done using a Luminograph I chemiluminescent imaging system (ATTO, Japan).

2.4 ELISA

The above-mentioned samples in the western blot analysis were used for ELISA. Affinity of produced antibodies was determined by indirect ELISA. ELISA plates were coated with 50 µl of 20 µg/ml HBsAg subtype *adr* (Fitzgerald Industries, North Acton, MA, USA) in coating buffer (Sera Care Life Sciences, Milford, MA, USA) and was incubated overnight at 4 °C. After coating, the plate was blocked with 1% BSA in PBS and incubated at 37 °C for 30 minutes. After washing with washing solution (MilliQ+ 0.05% Tween-20+ 154 mM NaCl) twice, 100 µl of sample was added to each well. The samples were incubated at 37 °C for 1 hour. The wells were washed again four times and incubated with goat anti-human IgG conjugated with HRP (Bethyl Laboratories, Montgomery, TX, USA) for 1 hour at 37 °C. Substrate solution was added to the wells. 100 µl ABTS peroxidase (Sera Care Life Sciences, Milford, MA, USA) was used as substrate for the HRP. After the color of the solution changed, 100 µl stop solution was added as buffer (Sera Care Life Sciences, Milford, MA, USA). The absorbance was detected at 405 nm wavelength using a microplate reader (Perkin Elmer Inc., Waltham, MA, USA).

3 Results and Discussion

3.1 Plasmid Construction

In this study, plasmid was constructed with the following steps: 1) cutting the DNA sequence from pEX-A2J2 plasmid and multiplication of the DNA sequence; 2) multiplication of pEHX1.2 and pELX2.2 plasmids in *E. coli*; 3) ligation of DNA fragments to be inserted in pEHX1.2 and pELX2.2 plasmids; 4) combination of pEHX1.2 heavy-chain and pELX2.2 light-chain linear plasmids, which produced pELC2+HC heavy-chain and light-chain plasmid. The results

from step 1-3 can be seen in the Supplementary Data. A schematic of plasmid construction can be seen in Figure 1. The result of ligation of the two vectors (pEHX1.2 heavy-chain and pELX2.2 light-chain) was confirmed by digestion with *EcoRI* and *SalI* restriction enzymes. An electropherogram of the cut products with the enzymes for colonies 1-5 is shown in Figure 2 and for colonies 6-10 in the Supplementary Data.

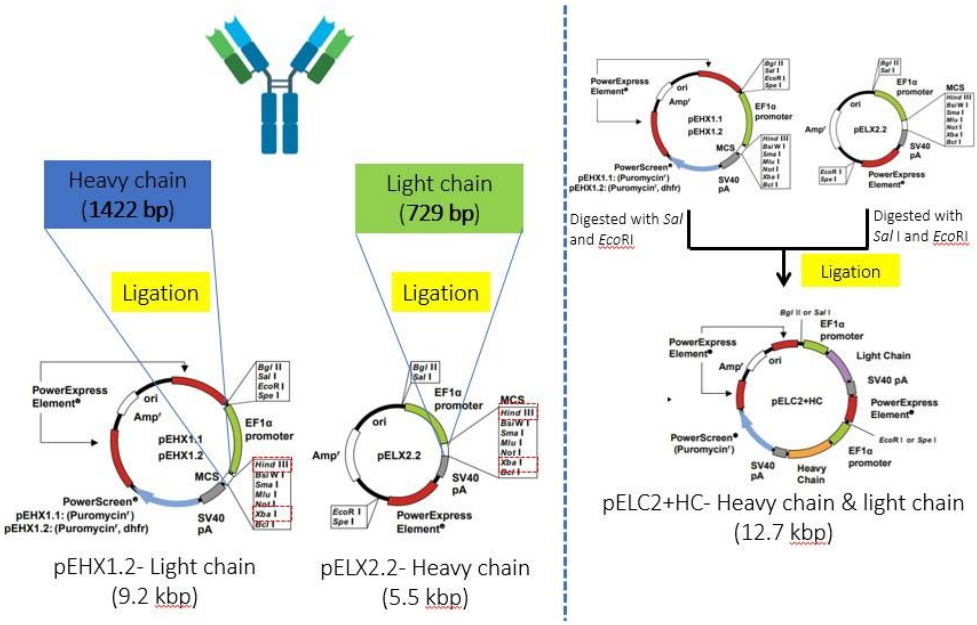

Figure 1 Schematic of plasmid construction.

Based on Figure 2(b), it was found that pELC2+HC heavy-chain and light-chain plasmid was successfully constructed in colonies 1, 3, and 9. This was indicated by the presence of two DNA fragment bands with a size of ±10 Kbp and ±3500 bp. The result was also confirmed by single digestion and only one band with size >10 Kbp obtained. The constructed plasmid from colony 1 was used for transfection to CHL-YN cells in the next step.

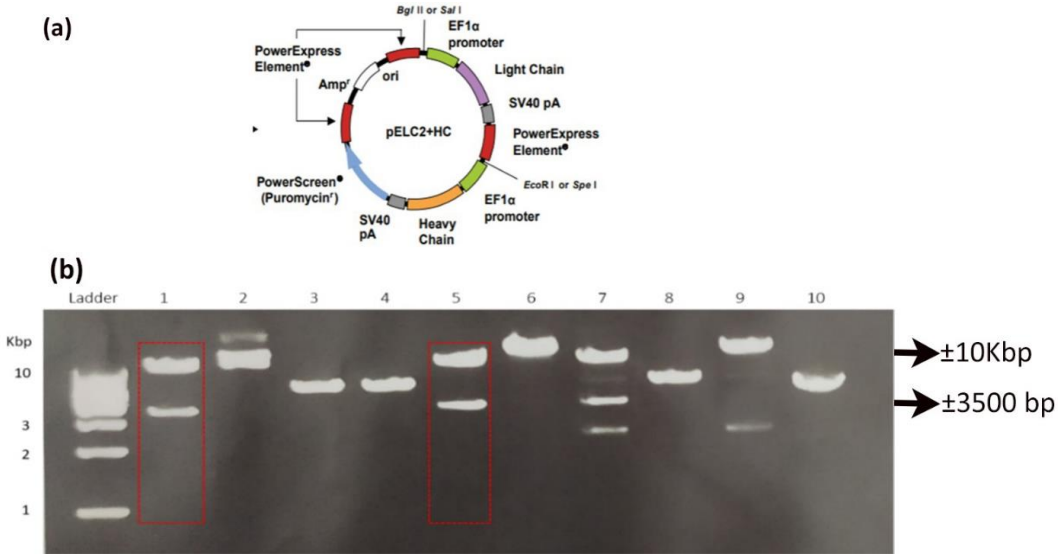

Figure 2 (a) Diagram of pELC2+HC from Mammalian PowerExpress System (Toyobo Co., Ltd., Japan). (b) Electropherogram of cut products with *EcoRI* and *SalI* restriction enzymes. Lane 1, 3, 5, 7, 9: double digestion with *EcoRI* and *SalI*. Lane 2, 4, 6, 8, 10: single digestion with *EcoRI*.

3.2 Transfection of pELC2+HC Heavy-Chain and Light-Chain Plasmid in CHL-YN Cells

Western blot analysis was used for detection of the antibodies produced by CHL-YN cells. The supernatant of transfected cells was collected at 48 and 72 hours. The result of the western blot analysis is shown in Figure 3. Based on Figure 3 it can be seen that there were two bands with sizes ±55 and ±28 kDa. These protein bands correspond to heavy chains and light chains, respectively. Both bands can be seen in the samples that were collected at 48 and 72 hours. Protein bands were not detected after 24 hours (data not shown), and after 48 and 72 hours, thin protein bands were detected in all transfected cells (data not shown). From this result, we can conclude that antibodies were transiently produced by the CHL-YN transfected cells.

In this study, we report the production of antibodies in a newly established CHL-YN cell line as an alternative host cell for CL4mAb production. Based on a previous report [13], CHL-YN cells successfully synthesized humanized IgG1 through stable expression. The advantage of using CHL-YN as host cells for antibody production is their ability to produce full and correctly structured antibodies. From our result, two protein bands with a size of ±55 and ± 28kDa were detected in the western blot analysis, indicating that heavy chains and light chains of the antibodies were successfully produced by the CHL-YN cells.

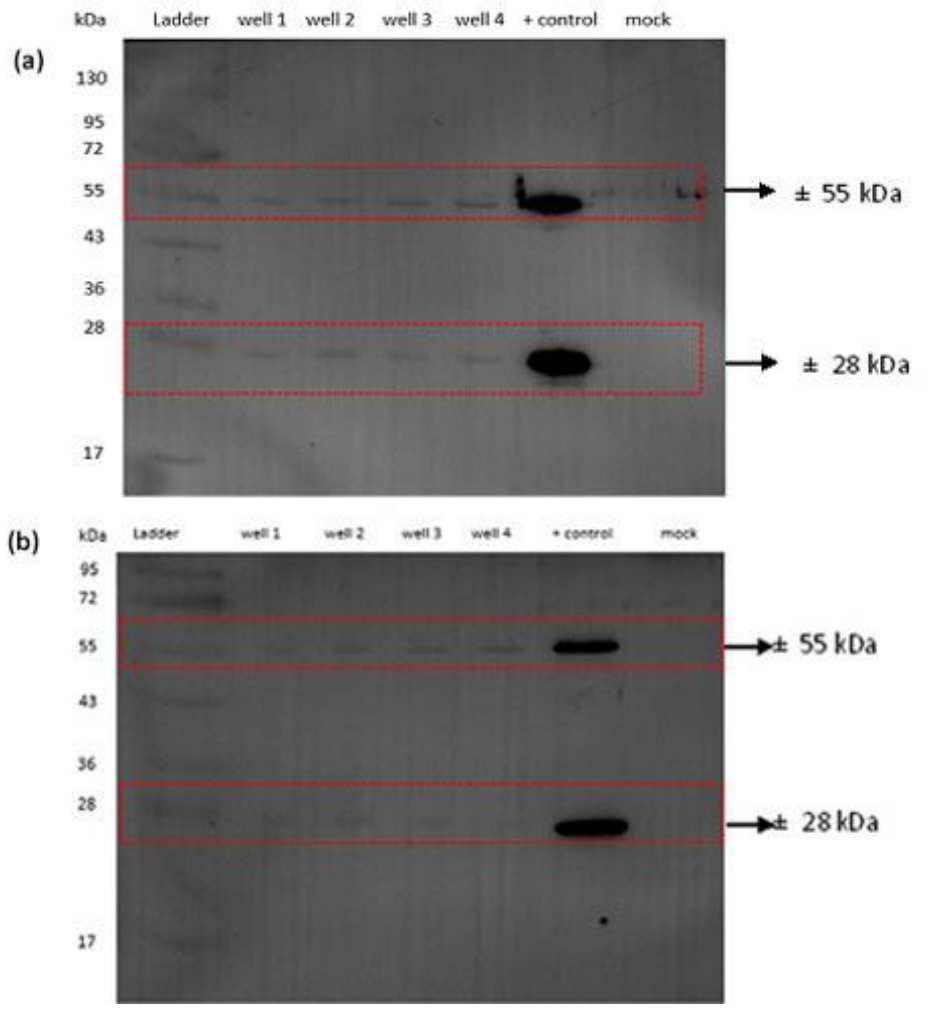

Figure 3 Antibody detection by western blot analysis. (a) Samples taken after 48 hours. (b) Samples taken after 72 hours. Two protein bands were detected with size ±55 and ±28 kDa, indicating heavy chains and light chains of the antibodies, respectively. Lane 1: protein marker; lane 2-4: supernatant from transfected CHL YN cells cultured in well 1 to well 4; lane 5: positive control (IgG1 producing CHL-YN cells); lane 6: mock vector (transfected with backbone plasmid).

3.3 Antibody Affinity Analysis with ELISA

ELISA was used to confirm the affinity of the antibodies produced with CHL-YN cells to the antigen HBsAg subtype *adr*. Indirect ELISA was used in this study. Samples from medium of the transfection cells were collected after 48 and 72 hours. The result of the absorbance reading is shown in Figure 4. The result of

the absorbance reading showed that the antibodies produced in the CHL-YN cells could recognize and bind with HBsAg subtype *adr*. All antibodies in the samples (wells 1, 2, 3, and 4) produced after 48 and 72 hours had the ability to recognize subtype *adr*. These results were confirmed by the negative control (transfection done without plasmid) and the mock vector (transfection done with backbone only), the samples (wells 1, 2, 3, and 4) had a higher absorbance value when compared to the negative control and the mock vector.

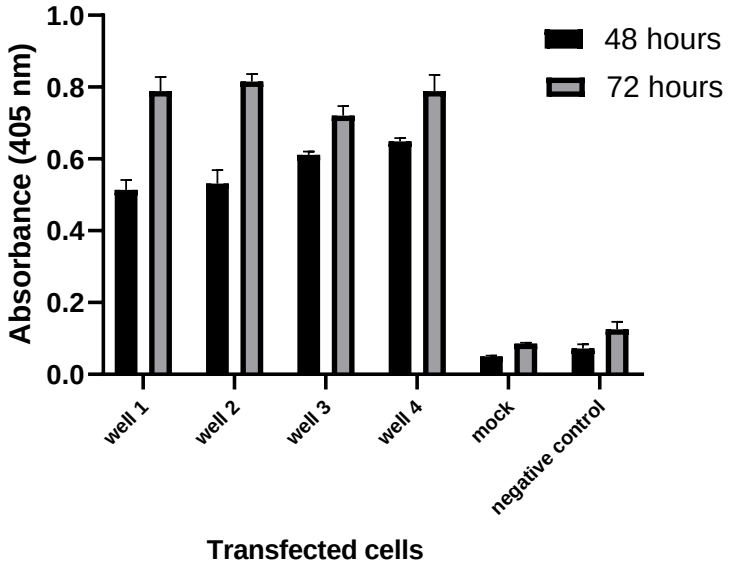

Figure 4. Result of absorbance reading. All antibodies in the samples (wells 1, 2, 3, and 4) produced after 48 and 72 hours had the ability to recognize subtype *adr* compared the mock vector and the negative control with average absorbance values, respectively (48 hours: 0.514, 0.531, 0.611, 0.649) and (72 hours: 0.789, 0815, 0.721, 0,789). Values are expressed as mean ± standard deviation (n = 3).

Antibodies for treatment of a disease should have the right function with the ability to recognize and bind to specific antigens. This ability can be possessed by the antibodies if the antibodies have the correct structure. The structure of the produced antibodies was predicted with the SWISS-Model software. The produced antibodies were predicted to have two identical heavy chains and two identical light chains. The predicted structure of the antibodies can be seen in Figure 5. From our result and in-silico analysis, it is predicted that the recombinant antibodies produced in CHL-YN cells may have a full structure that

is similar to IgG. Further analysis such as non-reducing western blotting is needed to confirm the structure of the antibodies produced in this experiment.

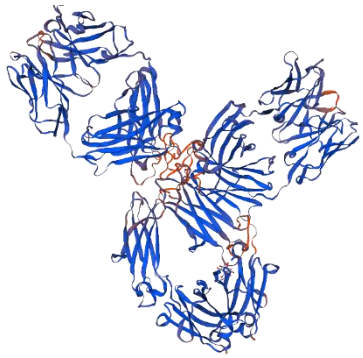

Figure 5. Predicted structure of antibodies produced by the CHL-YN cells. Analysis conducted with the SWISS-Model software.

The complex structure of the antibodies can be obtained by the formation of disulfide bonds through post-translation modification in endoplasmic reticulum (ER) [14]. Formation of disulfide bonds is very important in assessing antibody structure, stability, and functionality [15]. This is one of the advantages of using CHL-YN cells as host cells for antibody production compared with other systems. The *E. coli* expression system has a disadvantage as a host cell for antibody production due to the lack of post-translation modification ability [16]. In this study, formation of disulfide bonds was predicted by using the CYSPRED software. The result predicted that there are disulfide bonds in amino acid cysteine 45, 110, 156, 216, 235 in heavy chains and in amino acid cysteine 41, 115, 164, 220, 246, 249, 281, 341, 387, 445 in light chains. The data are shown in Table 1. Disulfide bonds between light chains and heavy chains of the antibodies occur at the last cysteine amino acid residue of the light chains and the fifth cysteine amino acid residue of the heavy chains [17]. This confirms the in-silico result, which showed the presence of disulfide bonds in the last cysteine amino acid residue (CYS236 light-chain) and the fifth cysteine residual amino acid (CYS220 heavy-chain) with a reliability value of 8. From our ELISA result, it was also seen that the antibodies could recognize HBsAg subtype *adr*, indicating that CHL-YN can produce antibodies that have the right structure and function. Further analyses, such as LC-MS/MS analysis, are needed to confirm the disulfide bonds presents in the antibodies.

Table 1. Prediction of sulfide bond sites in light chains and heavy chains of the antibodies.

Light chain		
Cysteine	Prediction	Reliability
CYS 45	Bonding State	8
CYS 110	Bonding State	8
CYS 156	Bonding State	4
CYS 216	Bonding State	8
CYS 236	Bonding State	8

Heavy chain		
Cysteine	Prediction	Reliability
CYS 19	Non-bonding State	9
CYS 41	Bonding State	8
CYS 115	Bonding State	4
CYS 164	Bonding State	8
CYS 220	Bonding State	8
CYS 240	Non-bonding State	9
CYS 246	Bonding State	1
CYS 249	Bonding State	0
CYS 281	Bonding State	4
CYS 341	Bonding State	4
CYS 387	Bonding State	4
CYS 445	Bonding State	7

4 Conclusions

pELC2+HC heavy-chain and light-chain plasmids were successfully constructed and transiently transfected on CHL-YN cells. Antibody IgG was secreted in the culture medium of the transfected cells. The secreted antibodies showed the ability to recognize and bind to HBsAg subtype *adr*.

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foundation, and partly by a Japan Society for the Promotion of Science KAKENHI Grant (JP21K04788).

6 Competing Interests

The authors declare no competing interests.

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