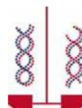
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In vitro generation of anti-hepatitis B monoclonal antibodies from a single plasma cell using single-cell RT-PCR and cell-free protein synthesis

Yunita Sabrina, Muhamad Ali,[1] and Hideo Nakano*

Laboratory of Molecular Biotechnology, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan

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Monoclonal antibodies (mAbs) are an effective tool in therapeutics and diagnostics. A novel approach called the single-cell RT-PCR-linked *in vitro* expression system (SICREX) enables the high-throughput generation and screening of mAbs from single B cells. In this paper, instead of using B cells, cDNAs were synthesized from single plasma cells of an immunized mouse spleen. The light chain (Lc) and the Fd portion of the heavy chain (Hc) genes of each cell were amplified separately and followed by overlapping PCR to add a T7 promoter, a ribosome-binding site, and a T7 terminator. The paired Lc and Hc genes were simultaneously expressed by an *Escherichia coli in vitro* transcription and translation system followed by ELISA to measure their affinity for the antigen. A Fab fragment with affinity against the antigen was obtained from plasma cells of an immunized mouse with hepatitis B surface antigen (HBsAg).

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[**Key words:** Single-cell; RT-PCR; Cell-free protein synthesis; Monoclonal antibodies; Hepatitis B]

Hepatitis B is one of the most frequent viral infections in humans in the world. Hepatitis B is an infectious disease that is caused by the hepatitis B virus (HBV) that attacks liver cells. The World Health Organization (WHO) has stated that 2 billion people have been infected with HBV worldwide; among these, more than 360 million people develop a chronic HBV infection and approximately one million die from the disease each year. The long-term consequences of chronic HBV infection are cirrhosis, liver failure, and hepatocellular carcinoma (1). Therefore, adequate prevention and effective treatments for hepatitis B are urgently needed.

The current approach for hepatitis B is based on interferon-α therapy in conjunction with lamivudine regimens. Although the pharmacokinetic and cytotoxic properties of these regimens have been improved, problems associated with these treatments including poor viral clearance, loss of response, and emergence of drug-resistant mutants have lowered the efficacy of the treatments (2). These problems and the urgency of hepatitis-related health problems continue to drive the development of new immunotherapeutic agents to combat hepatitis B. Currently, monoclonal antibodies (mAbs) are popular candidates for protection against infection and have been used successfully to treat respiratory syncytial virus infections (3).

Since mAbs are antibodies produced by a single clone of B lymphocytes, in contrast to polyclonal antibodies, mAbs are monospecific and homogenous (4). The long half-life, low toxicity, high affinity, and specificity of mAbs are only a few of the advantages that make them attractive potential therapeutic agents. Their wide application in detecting and identifying serum analytes, cell markers, and pathogenic agents has largely arisen through the exquisite specificity of these unique reagents (5).

Despite their utility, a method that can rapidly generate mAbs is not yet available. Over several past decades, following a report of hybridoma, a hybrid cell produced by the fusion of an antibody-producing lymphocyte with a tumor cell (6) has comprised the common mode of manufacturing mAbs. The hybridoma can continuously produce mAbs for years, but preparing the hybridoma involves a labor-intensive and multistep process limited by the constant risk of contamination, requirement of feeder cells, and genetic instability of the hybridoma (7). Newer methods to generate mAbs include display techniques such as phage display (8, 9), yeast surface display (10), ribosome display (11), and mRNA display (Chen, Y., 4th Annu. Conf. on Recomb. Antibody, USA, 2003). Display systems are used for the selection of coding elements from libraries in which the individual peptides or proteins as phenotypes are physically associated with their genetic material. These methods have the ability to handle large libraries and to evolve proteins through an iteration of random mutagenesis and selection (12). mAbs derived from a hybridoma are referred to as an antibody with a naturally original pair of a light chain (Lc) and a heavy chain (Hc). It has been stated that mAbs produced from single cells have a higher activity and specificity than those obtained from the random combinatorial library (13). A current application of display methods involves the separate amplification of the variable segments of heavy (VH) and light (VL) chains from pooled lymphocytes. Although antibody fragments with good affinity have been obtained by this way, it is unclear how often this approach

* Corresponding author. Tel.: +81 52 789 4143; fax: +81 52 789 4145.
E-mail address: hnakano@agr.nagoya-u.ac.jp (H. Nakano).
[1] Present address: Faculty of Animal Sciences, University of Mataram, Jl. Majapahit No. 62 Mataram, 83125, Indonesia.

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leads to the recovery of the original pairs of Lc and Hc expressed by individual B cells. In other words, the selected fragments do not contain the naturally occurring combinations of Lc and Hc and, therefore, do not represent their *in vivo* specificities and affinities (14).

An approach that could potentially be used to produce mAbs that have a correct pairing of Lc and Hc would rely on the use of single cell-based reverse transcription polymerase chain reaction (RT-PCR) to amplify an immunoglobulin gene, and subsequently introduce it into bacterial expression vectors. This technique is based on Burnett's clonal selection theory which proposed that each B lymphocyte produces only a single antibody (15). mAbs produced from single cells have better activity and specificity than those obtained from the random combinatorial library (13). A single cell-based RT-PCR is necessary to yield a correctly paired VH + VL Fab (16). Single-cell RT-PCR has been used for the amplification of antibody genes from various cells and resources (16, 17). However, in the previous methods, the RT-PCR product had to be cloned for expression analysis (18), which requires laborious work and time and is limited by the achievable number of cloning. More recently, the single-cell RT-PCR-linked *in vitro* expression (SICREX) method has been used for high-throughput monoclonal antibody screening (19, 20). The method was successfully used to obtain Fab fragments from the spleen cells of an immunized mouse as also from human peripheral blood.

In the present study, we used the SICREX method to bypass *in vivo* expression for rapid generation and screening of anti-hepatitis B mAbs from single plasma cells instead of B cells. The single-cell RT-PCR procedure is illustrated in Fig. 1. This method facilitates the high-throughput construction and screening of monoclonal antibodies.

MATERIALS AND METHODS

Immunization BALB/c mice were immunized intraperitoneally with 100 μg of plasma-derived HBsAg, after emulsification with complete Freund's adjuvant. HBsAg was kindly provided by Dr. Mulyanto (Mataram University). Mice were boosted twice with 50 μg of HBsAg after interval of 2 weeks and then after 10 days. After the third immunization, the sera were screened for the presence of protective antibodies to HBsAg. Three days after the second boost, the spleen were harvested and manually disaggregated in phosphate buffered saline (PBS). The present study was approved by the committee of animal experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Isolation of single plasma cells The splenocytes were collected at 1000 rpm for 3 min at 4 °C, and washed once in PBS. The solution of the cells was incubated with MACS super-paramagnetic microbeads conjugated to the monoclonal anti-mouse CD 138^{+} antibody, according to the manufacturer's instructions (Milteny Biotech, Germany) and passed through a magnet. The isolated CD 138^{+} plasma cells were counted under a microscope (NIKON, Japan). Then the plasma cells were diluted into 1 cell per PCR tube, and immediately used for cDNA synthesis or stored at −80 °C for later use.

Single-cell RT-PCR Reverse transcription and subsequent PCR amplification were carried out using the GeneAmp^{R} PCR system 9700 (PE Applied Biosystems, USA). The first-strand cDNA of each cell was synthesized by using the SUPERSCRIPT II First-Strand Synthesis System for RT-PCR (Invitrogen, USA), according to the manufacturer's protocol. The reaction mixture contained 0.1 μM of each primer (Ck-J1, CH-IgG1-1, CH-IgG2A-1, CH-IgG2B-1, and CH-IgG3-1) to yield cDNA for all of these classes. A control synthesis reaction was simultaneously performed without plasma cells to test for contamination.

Briefly, the resulting cDNAs were used as templates for two-round PCR to obtain Lc and Hc genes separately. The cDNAs were amplified using the cDNA-specific primer in the first round of PCR. The Lc gene was amplified using one set of cDNA-specific primers (Vk-M2 and Ck-His6M2); a mixture of 6 primers (VH-M2, V′H-M2, CH-IgG1-M2, CH-IgG2A-M2, CH-IgG2B-M2, and CH-IgG3-M2) was used for Hc gene amplification. The amplification reaction was performed in a total volume of 5 μl with 0.25 U of *Taq* DNA polymerase (Takara Bio Inc., Japan), 0.2 mM of each dNTP, 0.05 μM of each primer, 0.5 μl of the cDNA mixture, and the reaction buffer using the following PCR program: 3 min at

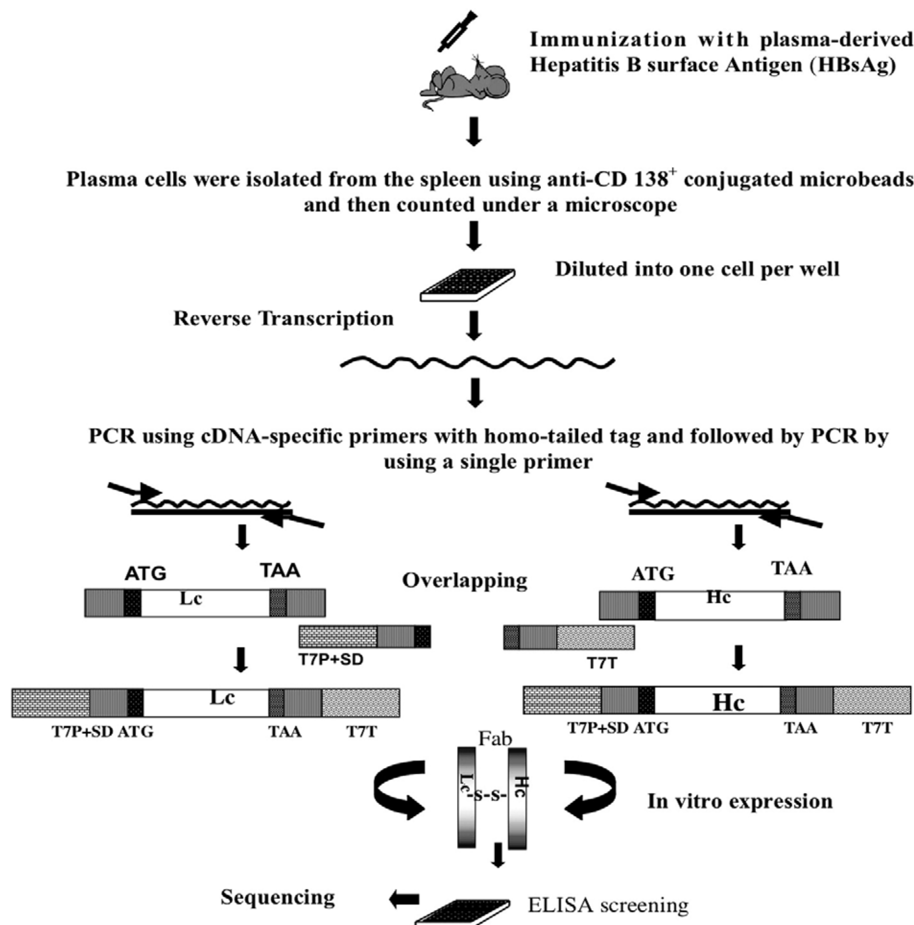

FIG. 1. Schematic illustration for the generation of a Fab fragment by single-cell RT-PCR-linked *in vitro* expression.

TABLE 1. Oligonucleotides used in single plasma cell RT-PCR.

Description	Name	Sequence
Ck-constant regions	Ck-J1	5′-TTAACACTCATTCCTGTTGAA-3′
CH-constant regions	CH-IgG1-1	5′-AATTTTCTTGTCCACCTTGGT-3′
	CH-IgG2A-1	5′-AATTTTCTTGTCCACCTTGGT-3′
	CH-IgG2B-1	5′-AAGTTTTTTGTCCACCGTGGT-3′
	CH-IgG3-1	5′-GATTCTCTTGATCAACTCAGT-3′
PCR		
VH-framework-1 region	VH-M2	5′-ATTAGATAAGAAGGAGATTATTGAATG(C/G)A(A/G)GT(A/C/G/T)(A/C)AGCTG(C/G)AG(C/G)AGTC-3′
	V′H-M2	5′-ATTAGATAAGAAGGAGATTATTGAATG(C/G)A(A/G)GT(A/C/G/T)(A/C)AGCTG(C/G)AG(C/G)AGTC(A/T)GG-3′
CH-constant regions	CH-IgG1-M2	5′-ATTAGATAAGAAGGAGATTATTGATTAAATTTTCTTGTCCACCTTGGTGCTGCT-3′
	CH-IgG2A-M2	5′-ATTAGATAAGAAGGAGATTATTGATTAAATTTTCTTGTCCACCTTGGTGCTG-3′
	CH-IgG2B-M2	5′-ATTAGATAAGAAGGAGATTATTGATTAAAGTTTTTTGTCCACCGTGGTGCTG-3′
	CH-IgG3-M2	5′-ATTAGATAAGAAGGAGATTATTGATTAGATTCTCTTGATCAACTCAGTCTTGCT-3′
Vk framework-1 region	Vk-M2	5′-ATTAGATAAGAAGGAGATTATTGAGA(C/T)ATTGTG(A/C)T(C/G)AC(A/C)CA(A/G)(A/T)CT(A/C)CA-3′
Ck-constant regions	Ck-His6M2	5′-ATTAGATAAGAAGGAGATTATTGATTAGTGGTGGTGGTGGTGGTGACACTCATTCCTGTTGAAGCT-3′
Single primer	SP2	5′-ATTAGATAAGAAGGAGATTATTGA-3′

94 °C; 32 cycles of 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C; and 7 min at 72 °C. Then, 0.5 μl of the first-round PCR product was amplified using the single primer. The amplification reaction was carried out in a total volume of 5 μl with 0.25 U of *Pfu* Turbo DNA polymerase (Stratagene, USA), 0.125 mM of each dNTP, 0.5 μM of SP2 primer, and the reaction buffer using the following PCR program: 3 min at 94 °C; 65 cycles of 10 s at 96 °C, 10 s at 50 °C, and 45 s at 72 °C; and 7 min at 72 °C. PCR was performed in 96-well PCR plates, which allowed the fast and efficient handling of large numbers of the samples.

Design of PCR construct for *in vitro* expression The T7 promoter and T7 terminator fragments were amplified from pRSET-B vector (Invitrogen, USA). The T7 promoter fragment was amplified using the primer T7Pf (5′-CGCCTGG-TATCTTTATAGTCCTGT-3′) and T7Pr (5′-ATAA TCTCCTTCTATCTAATAACAAAA-TTATTTCTAGAGGGAAACCG-3′), and for the T7 terminator fragment using the primer T7Tr (5′-TAATCAATAATCTCCTTCTTAT CTAATTCCGGCTGCTAACAAAGCCCG-3′) and T7Tf (5′-TGACGGGGAAAGC CGGCGAA-3′). The second round PCR product of Lc or Hc was inserted between the 2 DNA fragments so obtained by overlapping PCR to generate full-length DNA fragments suitable for cell-free protein synthesis. The overlapping PCR was carried out in 15 μl volume with 0.75 U of the *Ex Taq* DNA polymerase (Takara Bio Inc., Japan), 0.2 mM of each dNTP, 0.15 μl of each DNA fragment, and a reaction buffer using the following PCR program: 3 min at 94 °C, 10 cycles of 30 s at 94 °C, 20 s at 48 °C, and 60 s at 72 °C. Then, 15 μl of the overlapping extension mixture was adjusted to 30 μl by the recommended reaction buffer containing 1.5 U of the *Ex Taq* DNA polymerase, 0.2 mM of each dNTP, 0.5 μM of each In-F (5′-CCAATA CGCAAACCGCCTCTCC-3′) and In-R primers (5′-TACAGGGCGCGTCCCATTC G-3′) using the following PCR program: 3 min at 94 °C, 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, and 7 min at 72 °C.

Cell-free protein synthesis The cell-free reactions were carried out in 30 °C for 1 h. In this procedure, 3 μl each of overlapping PCR product was transcribed and translated in 30 μl of mixture (56.4 mM Tris-acetate, pH 7.4, 1.22 mM ATP, 0.85 mM each of GTP, CTP, and UTP, 50 mM creatine phosphate, 0.5 mM each of all 20 unlabeled amino acids, 4% polyethyl glycerol 6000, 34.6 μg/ml folinic acid, 0.17 mg/ml *Escherichia coli* tRNAs, 35.9 mM ammonium acetate, 10 mM $Mg(OAc)_2$, 100 mM KOAc, 10 μg/ml rifampicin, 0.15 mg/ml creatine kinase, 7.6 μg/ml T7 RNA polymerase, 28.3% [v/v] S30 extract, 0.1 mM GSH, 1 mM GSSG, and 36 μg/ml protein disulfide-isomerase (PDI). PDI from *Humicola iosolens* (21), which was kindly provided by Toyota Central Research and Development Laboratories (Japan), was used at a final concentration of 36 μg/ml. At the same time, the reaction mixture without the overlapping product was used for control reactions.

Enzyme-linked immunosorbent assay (ELISA) The activity of the Fab fragments against HBsAg was examined using ELISA. Each well of 96-well microtiter plates (Nunc, Denmark) was coated with 50 μl of 5 μg/ml HBsAg. After incubation at 4 °C overnight, the wells were blocked using 25% Block Ace (Dainihon Pharmaceuticals, Japan) in distilled water and incubated at 37 °C for 30 min, and then washed twice with a washing solution (10% Block Ace, 0.05% Tween-20). The cell-free product as the first antibody was diluted 5-fold with PBS (pH 7.4). Then, 50 μl of this solution was added into the plate, incubated at 37 °C for 2 h, and washed twice with the same washing solution. Subsequently, 100 μl of the second antibody was added and incubated for 2 h at room temperature. Anti-mouse IgG (H + L) specific peroxidase conjugate (Calbiochem, Germany) was used as the second antibody. After washing twice, a solution containing *o*-phenylenediamine (Wako Pure Chemical Industries, Japan) and H_2O_2 were used as the substrate for the peroxidase reaction. Then, the plate was incu-bated at 37 °C for 10–30 min or until the solution became colored; after adding 2 M H_2SO_4 to stop the color reaction, the absorbance was measured at 492 nm.

Sequence analysis The Lc and Hc genes were cloned into a pGEMT-easy vector (Promega, USA) and then sequenced with Thermo Sequenase™ II BigDye terminator Ver.3 sequencing premix kit (Amersham Pharmacia Biotech, Sweden) by an ABIPR-ISM™ 310 Genetic analyzer (Applied Biosystems, USA), according to the recommended protocol. The germline gene was determined using the IgBLAST database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/igblast). Identification of complementary determining regions (CDRs) was performed by examining the sequence based on the Kabat definition (22).

RESULTS

Amplification of Lc and Hc genes from single plasma cells The primers used in the single-cell RT-PCR have been previously described (19) and are listed in Table 1. For the amplification of the V regions, the FR1 regions from VL and VH were chosen as target sequences for the 5′ primers. The 3′ primers anneal to the highly conserved C-terminal sequences of the constant region, which minimizes the mismatch bias during PCR amplification. Also, these forward primers contain wobble bases in up to 6 positions at the 3′-end to reduce the primer specificity that permits a high number of mismatches. With these designs, we can use the minimum number of degenerated primers to cover the Ig gene.

Since a mouse expresses 95% of κ-isotype Lc, λ-Lc was ignored in the primer designs (23). For Hc, only IgG, which has a high antigen-binding activity and is less prone to degradation, was targeted. These primers were designed to have a homotag sequence at both ends. Using the homotag primer, a PCR product with homopriming sequences at both ends would be produced, and this would subsequently facilitate further amplification using a homotag-specific primer (SP2).

The first-strand cDNA from plasma cell was synthesized using the SS II First-Strand Synthesis System, the resulting cDNAs were subsequently

FIG. 2. Agarose gel electrophoresis analysis of the RT-PCR product obtained from a single plasma cell of an immunized mouse. The results of the amplification of the Lc (A), and Hc (B) genes are partly shown.

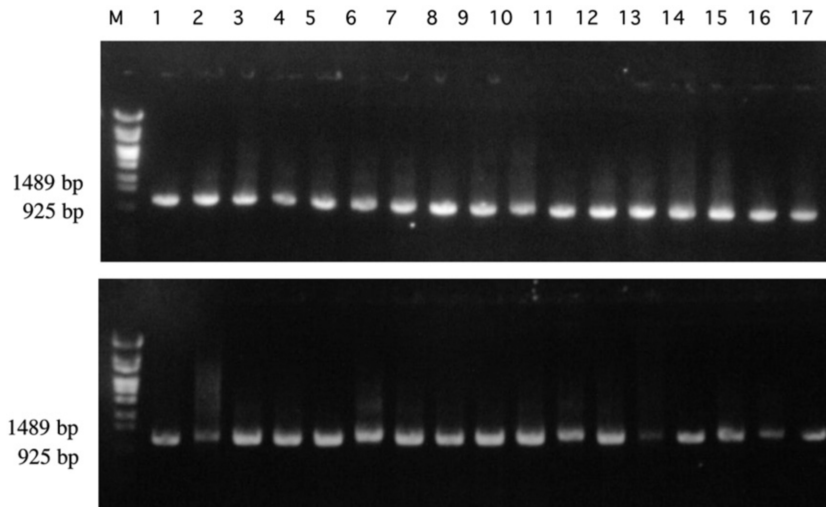

FIG. 3. Agarose gel electrophoresis analysis of overlapping PCR products obtained from a single plasma cell of an immunized mouse. The amplification result of pairing Lc (upper panel) and Hc (lower panel) genes was shown.

used as templates for the first round of PCR to obtain Lc and Hc genes separately using the established PCR conditions mentioned in the Materials and methods section. The amplification results from plasma cells are shown in Fig. 2; 60 Lc genes and 20 Hc genes were obtained from 96 wells, and 17 wells gave a complete pair of Lc and Hc genes (17.7% recovery ratio). The pairing of Lc and Hc genes indicated that both genes were amplified from the same cell ensuring the correct original pairing of the antibody genes. Some wells may have contained no cells, because of which neither Lc nor Hc was amplified.

In order to make the Lc and Hc amplified product ready for cell-free protein synthesis, it is considered that a promoter, Shine Dalgarno sequence, and a terminator sequence should be added (24). Therefore, the amplified Lc or Hc was joined by using the overlapping PCR technique with DNA cassettes for the T7 promoter (PT7) containing the Shine Dalgarno sequence and the T7 terminator (T7T). When these PCR products are mixed, denatured, and re-annealed, the strands that have the matching sequences at their ends overlap and act as primers for each other. An extension of this overlap by *Ex Taq* DNA polymerase produces a gene construct ready for cell-free protein synthesis (PT7-Lc-T7T and PT7-Hc-T7T). The result of Lc and Hc overlapping PCR is shown in Fig. 3. For high-throughput functional screening, these constructs can be directly expressed in a cell-free protein synthesis system (25). This step made this method different from the previous methods of mAbs production using single-cell RT-PCR, since in the previous experiments the Lc and Hc genes were cloned into an expression vector before screening or generation. Omitting the necessity for gene cloning makes this method suitable for high-throughput mAbs production.

Expression and screening of Fab fragments In an attempt to ensure the production of a naturally correct pairing of antibody genes, the overlapping products of paired Lc and Hc genes were placed into the same reaction tube and then transcribed and translated in cell-free protein synthesis at 30 °C for 1 h. Since the cell wall barrier is not present in cell-free reactions, the reaction environment can be directly controlled and allows manipulation of the reaction environment to promote folding of disulfide bonded proteins (26). When the Lc and Hc are simultaneously transcribed and translated in the *E. coli* cell-free protein synthesis system under naturally oxidizing conditions, Lc and Hc have a natural tendency to connect to each other by an interchain disulfide bond, resulting in the formation of a Fab fragment (27). PDI has been shown to be associated with immunoglobulin production *in vivo*; further, it is known to be involved in their folding *in vitro*. PDI changes the redox dependence of the reaction; in the presence of PDI, the formation of the correct disulfide bonds is possible at higher oxidizing conditions as compared to spontaneous reactions (28).

An important point of this technology is that it allows the direct screening of the *in vitro* synthesized product for the identification of specificity and desired characteristics. In this study, ELISA was performed by using *in vitro* synthesized Fab fragments against HBsAg in order to determine the Fab fragment affinity for the antigen.

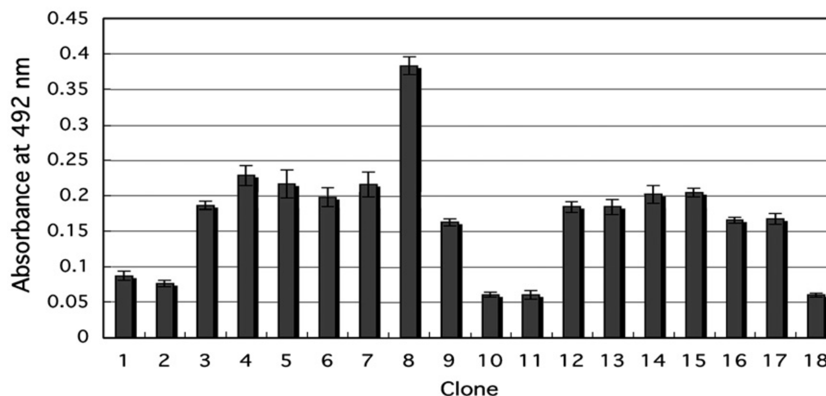

FIG. 4. ELISA against HBsAg of Fab from single plasma cells (nos. 1–17) and control (no template, 18). These Fabs were synthesized in an *E. coli* cell-free protein synthesis system. Samples were taken at 60 min after the start of incubation.

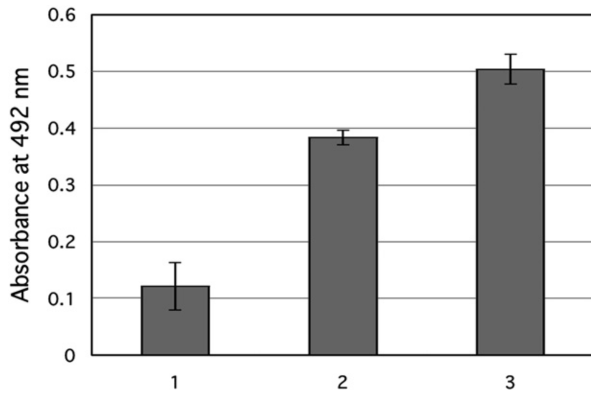

FIG. 5. ELISA of clone no.8 Fab with no coating antigen (1), coating with HBsAg (2), and coating with anti his (C-term) antibody (3). Fab was synthesized in an *E. coli* cell-free protein synthesis system. Samples were taken at 60 min after the start of incubation.

As seen in Fig. 4, the ELISA signal of clone no. 8 Fab is higher than that of the other clones. This result strongly suggests that the synthesized Fab was capable of binding the HBsAg. Furthermore, since there is a his6-taq in the C-terminal of Lc, we conducted ELISA of clone no.8 Fab against anti his (C-terminal) antibody. As seen in Fig. 5, the ELISA signal with anti his (C-terminal) antibody as coating antigen is 4 times higher compared to the signal with no antigen coating.

DNA sequencing of the positive clone The Lc and Hc genes of clone no. 8 were sequenced, analyzed, and compared with the mouse germline gene. The sequence analysis shows that the Lc consists of 666 nucleotides encoding 222 amino acid residues (Fig. 6), containing 4 framework regions (FRs) and 3 complementarity-determining regions (CDRs). The Lc sequence of clone no. 8 was found to be 98.6% identical to that of the 19–25 germline gene (GenBank accession no. AJ235962). The Hc sequence of clone no.8 which consists of 645 nucleotides encoding 215 amino acid residues (Fig. 7), contains 4 framework regions (FRs) and 3 complementarity-determining regions (CDRs). The Hc sequence of clone no. 8 was found to be 94.6% identical to that of the V 130.2 germline gene (GenBank accession no. AF455976).

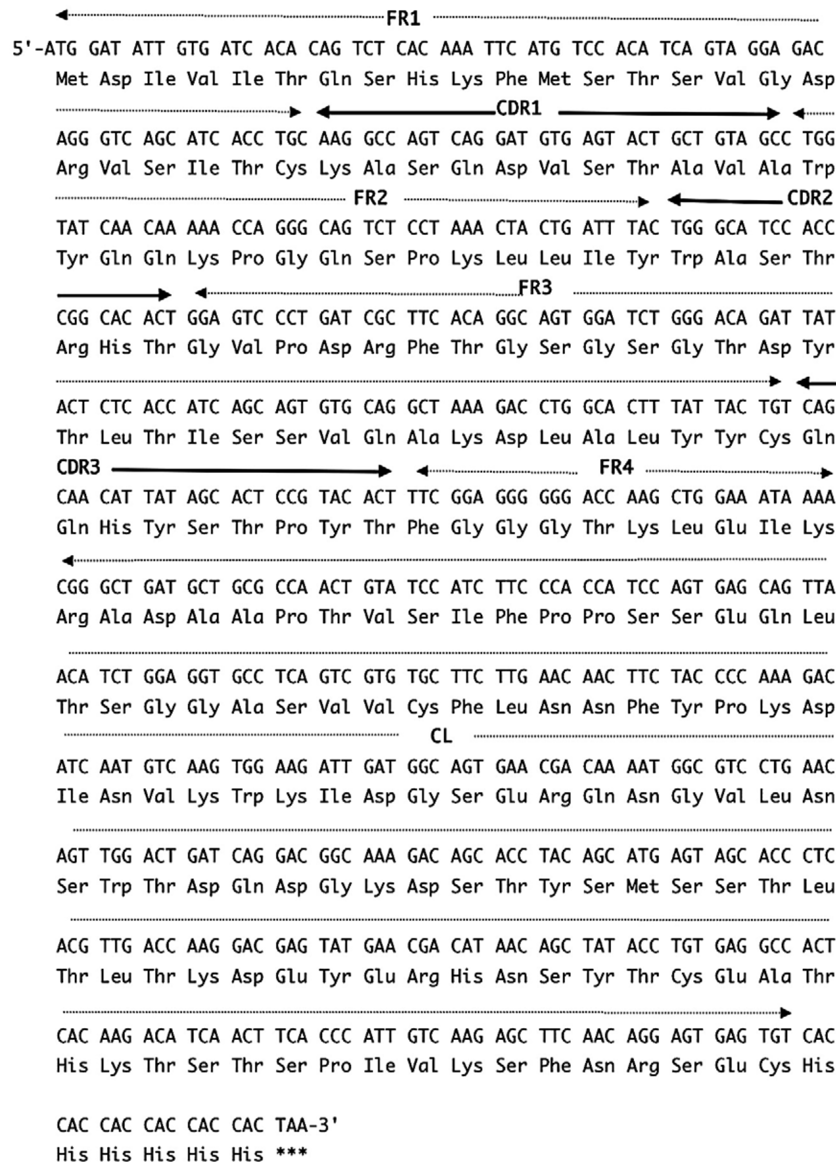

FIG. 6. Lc sequences of clone no.8.

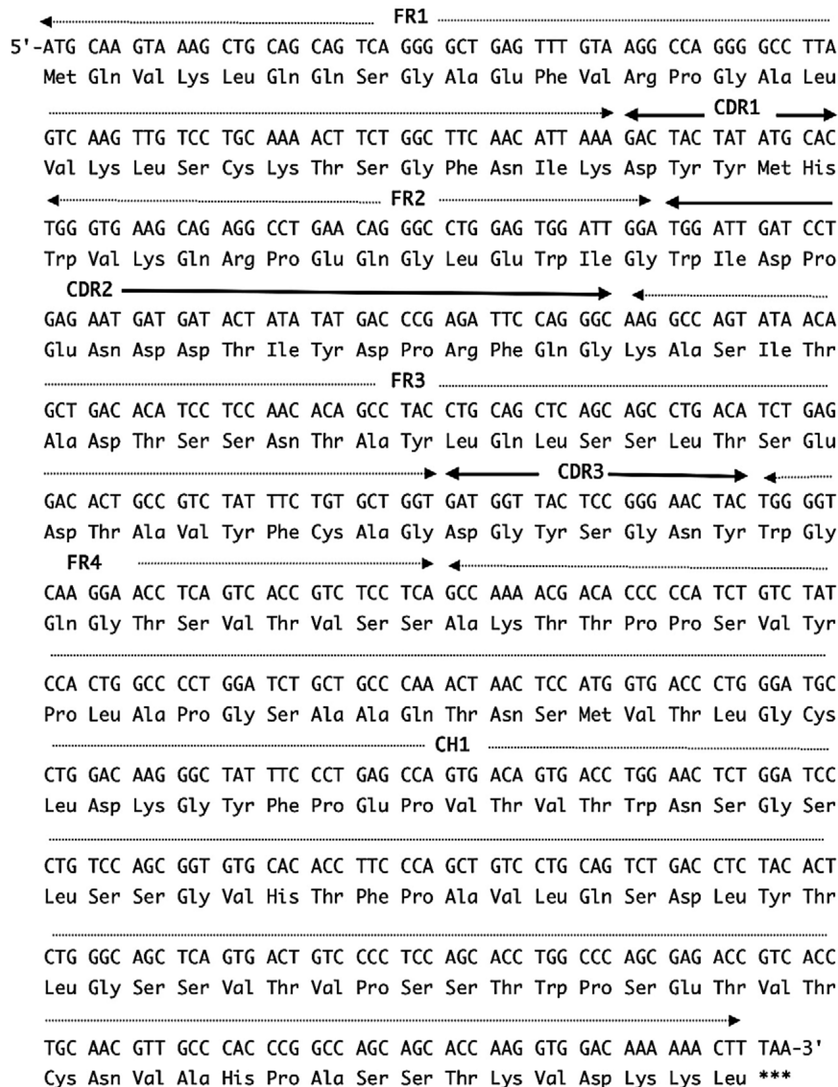

FIG. 7. Hc sequences of clone no.8.

Results of the CDR identification indicated that the CDR-L sequence had characteristics with those of the CDR-L sequence in the Kabat definition. The CDR-L1 sequence starts from the 24th residue next to the 23rd C and residue after is always W, similar to the Kabat definition. In addition, the consistency of the CDR sequence was also observed for the CDR-L2 and CDR-L3 with small differences. The result also indicated that the CDR-H sequence had characteristics with the CDR-H sequence mentioned in the Kabat definition. The CDR-H1 sequence starts from the 31st residue, the 9th residue before CDR-H1 is always C and residue after is always W, similar to the Kabat definition. The consistency of the CDR sequences was also observed for CDR-H2 and CDR-H3 with small differences.

DISCUSSION

In this research, we employed the SICREX method to produce mAbs from a single cell, based on Burnet's clonal selection theory, which considers that each B lymphocyte is committed to producing only a single structure (7, 29). This is achieved by the expression of only one of the available alleles a process termed allelic exclusion that ensures that a single B lymphocyte secretes an antibody with only one type of an antigen-binding site. The one-cell-one-antibody theory allows the construction of mAbs by the amplification of an Ig gene from a single cell by the RT-PCR technique.

Using the SICREX system, we generated the Fab fragment of mAbs against HBsAg. Fabs have the same binding characteristics as the parent antibody; however, because of their smaller size, they have greatly improved tissue penetration and clearance (30). Moreover, Fabs are attractive for use as therapeutics due to their monovalent antigen binding, lack of innate effecter functions, and ability to be produced in a range of large-scale expression hosts such as *E. coli*.

The *in vitro* synthesized protein of Lc, Hc, and Fab fragment of clone no. 8 were analyzed by SDS-polyacrylamide gel electrophoresis with and without beta mercaptoethanol followed by autoradiography (data not shown). We found that Lc and Hc were expressed equally, but unfortunately the Fab fragment in non-reducing condition could not be detected. It seems that the stability of the disulfide bridge between Lc and Hc were different in each antibody since the bond in the Fab fragment of 6D9, a mouse antibody, was detected in the same condition (27). It is said that an inter chain disulfide bond which connects Lc and Hc in a Fab fragment is susceptible to reduction (31) and beta elimination because it is not protected unlike intra chain disulfide bonds (32). Rudikoff and Pumphrey (33) stated that the presence of a disulfide bond in the variable region is not critical to the proper folding and function (antigen binding) of variable region. We

believe that even without the experimental detection of the disulfide bridges, the variable region of the Fab fragments produced in our *in vitro* system still gains their function and binds to its antigen. Nevertheless, the condition of the cell-free protein synthesis system would be improved in order to make a stable inter chain disulfide bond of every Fab fragment in near future.

In the single-cell-based RT-PCR method to amplify immunoglobulin genes, the specific amplification of Lc and Hc genes is important since the immunoglobulin genes are highly diverse in their amino acid composition and nucleotide sequences. The important factors for successful amplification are the initial amount of template, primer design, and the conditions used for RT-PCR. Since the amount of RNA present in single cells is very limited, i.e., only 0.1–1 pg (34), this amount may not be sufficient to serve as a template for RT-PCR and can reduce the efficiency of PCR amplification. To overcome this problem, we used as a template for RT-PCR plasma cells which are known to express up to 1000-fold more Ig mRNA per cell than B cells (16). Plasma cells are highly specialized, terminally differentiated cells that produce tremendous quantities of single soluble antibody molecules that mediate humoral immunity (35).

In an attempt to increase the amplification rate, we used *Taq* DNA polymerase in the first round of PCR since this polymerase lacks 3′-exonuclease activity and tolerates mismatches between highly degenerate primers and templates much better than do other DNA polymerases (36). In addition, a high-fidelity DNA polymerase, *Pfu* Turbo DNA polymerase (Stratagene), was used for the second round of PCR amplification. The use of this polymerase would significantly reduce the number of mutations in the amplified product. Also, all the primers for PCR were designed to have a homotag sequence at their ends. Using a homotag primer, a PCR product with homopriming sequences at both ends would be produced, and this would subsequently facilitate further amplification using a homotag-specific primer. This method greatly decreased the possibility of formation and subsequent accumulation of primer–dimers that inhibit the amplification of the target template (37) because of the “pan-handle” structure formation (38).

The amplification rate of the Hc gene was lower compared to that of Lc gene; a possible reason for this problem is the higher diversity of the Hc region compared to that of Lc region, because of which the primers used in PCR amplification cannot cover the entire mouse Hc immunoglobulin repertoire. Other research has also noted difficulties in the Hc amplification (39). There have been numerous descriptions of primer designs for PCR amplification of mouse Ig genes in recent years; however, these still represent only a small fraction of the highly diverse sequences of mouse Ig gene. Therefore, much effort is necessary for designing a universal primer that permits the amplification of all IgG.

Single-cell RT-PCR is a highly sensitive technique, and great care should be taken during the experiment to avoid mRNA cross contamination and degradation since even a very small amount of contamination could be amplified. Unfortunately, compared to B cells, plasma cells rapidly undergo apoptosis *ex vivo* and contain a large amount of mRNA, which can be a source of contamination (16); however, this can be minimized by the maintenance of low temperature and quick and gentle handling in all the steps of the experiment. All equipment and solutions should be RNase free; other procedures for minimizing contamination include the irradiation of all equipment by UV light 30 min before use and the consistent use of a filter tip for distributing reagents.

The SICREX system has several advantages. First, this method is based on a single cell, which ensures that the mAbs produced have a better activity and specificity than those obtained from the random combinatorial library. Second, it allows the direct screening of the *in vitro* synthesized product for the identification of specificity and the desired characteristics. Third, the omission of *in vivo* expression resulted in a rapid generation of the monoclonal antibodies. As compared to the hybridoma method, which requires 2–3 months for completion, the generation of mAbs using this method requires only 2 days.

In conclusion, having successfully expressed the Fab fragment from single plasma cells in a rapid and simple manner, we consider the SICREX method to be convenient and applicable for high-throughput generation and the screening of naturally paired mAbs.

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