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Rapid and reliable hybridoma screening method that is suitable for production of functional structure-recognizing monoclonal antibody

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Monoclonal antibodies are extremely valuable functional biomaterials that are widely used not only in life science research but also in antibody drugs and test drugs. There is also a strong need to develop high-quality neutralizing antibodies as soon as possible in order to stop the rapid spread of new infectious diseases such as the SARS-CoV-2 virus. This study has developed a membrane-type immunoglobulin-directed hybridoma screening (MIHS) method for obtaining high-quality monoclonal antibodies with high efficiency and high speed. In addition to these advantages, this paper demonstrates that the MIHS method can selectively obtain monoclonal antibodies that specifically recognize the functional structure of proteins. The MIHS method is a useful technology that greatly contributes to the research community because it can be easily introduced in any laboratory that uses a flow cytometer.

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The technique for producing monoclonal antibodies (mAbs) was invented by Köhler and Milstein (1) in 1975, and many mAbs have been produced since then (2). By utilizing the mAb production technique, it is possible to industrially produce a large amount of a uniform antibody with high binding specificity for a target molecule. Therefore, mAbs that are useful as functional molecules are used not only in basic science but also in pharmaceuticals, test drugs, biosensors, and so on. In recent years, sales of mAbs-based drugs have dominated small and medium-sized drugs, and they are expected to reach market revenues of approximately $218.97 billion by the end of 2023 (2).

There are two types of mAbs: antibodies that recognize the linear epitope of the primary protein structure and antibodies that recognize secondary, tertiary, and higher structures (3). In life science research, the use of antibodies that recognize linear epitopes includes methods such as ELISA and Western blotting. However, applications using this type of antibody are limited, whereas structure recognition antibodies have a wide range of applications such as immunoprecipitation, immunostaining. When a mAb is used as a drug, a structure recognition antibody is particularly important. Since the target protein in the body functions by forming an appropriate three-dimensional structure, it is crucial that the antibody recognizes the three-dimensional structure of the protein in order to pharmacologically inhibit the function. Therefore, it is necessary to replace linear epitope mAbs with stereospecific mAbs (2). In recent years, antibody drugs for SARS-CoV-2 virus (4), chikungunya virus (5,6), and HIV-1 virus (7) have been developed, but the focus has been on developing widely neutralizing antibodies that target conformational epitopes (5,6). However, most mAbs obtained by classical hybridoma technology recognize linear epitopes, and there is a strong demand for technological development to selectively obtain structure-recognizing antibodies.

The MIHS (membrane-type immunoglobulin-directed hybridoma screening) method (5) utilizes the fact that antibody-secreting hybridomas express membrane-type immunoglobulins (mlg), which have the same antigen-binding specificity as the secreted antibodies, on the cell membrane. In the MIHS method, a fluorescently-labeled peptide antigen is bound to mlg, hybridoma cells secreting the target antibody are selected by a flow cytometer (FCM), and each cell is seeded on a 96-well plate. This allows the most labor-intensive and time-consuming screening and cloning steps to be completed in a day. In a previous report using peptide antigens as screening antigens, some clones seemed to preferentially recognize the conformation (8). In this method, since the screening antigen is in contact with mlg in the medium, it was expected that a clone could be obtained that recognized the antigen protein with a folded structure in the solution. This hypothesis was verified by using a recombinant EGFP protein with a functional structure as a screening protein. This study has developed an efficient method for obtaining functional structure-specific mAbs using a recombinant protein expressed in Escherichia coli with an EGFP fluorescent tag and a functional structure as a screening antigen.

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Sorting His-EGFP recombinant protein (2 nmol) was mixed with hybridoma in 1 mL RPMI 1640 medium supplemented with 10% newborn calf serum (Biowest, Nuaille, France), and the cells were incubated for 2 h at 37 °C in 5% CO₂. The cells were centrifuged at 12,000 rpm at 800 rpm for 3 min, washed three times, and finally resuspended in 500 μL RPMI 1640 medium. The hybridoma cell sorting was carried out as described previously (7).

**Hybridoma screening by western blotting**

A second screening was performed using western blotting analysis. The protein samples prepared from E. coli that stably expressed His-EGFP or myeloma-expressed EGFP (1 × 10⁶ cells) were boiled, resolved by 12% SDS-PAGE, and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P SQ, Millipore-Millipore, Darmstadt, Germany). The membrane was divided into strips. The strips were blocked overnight with 5% skim milk in TBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM NaH₂PO₄ with a pH of 7.2). After 3 days of the final immunization, the mice were sacrificed, and the spleenocytes were collected in PBS. The procedures for cell fusion and purification of hybridoma were the same as those in the previous paper (8). The protocols for the animal experiments were reviewed and approved by the Ethics Committee on Animal Experiments of Yokohama National University.

**Material and methods**

Plasmid expression and purification of the antigen protein. **The vector maps of the E. coli expression vectors used in this study (EGFP-BirHis-TE23 (+), and His-EGFP pColIII)** are shown in Fig. S1. The expression vector was transformed into E. coli BL21(DE3) pLysS and seeded on an LB agar plate containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol. After culturing at 37 °C for 16 h, single colonies from the plate were cultured in 1 L of LB medium containing 30 μg/mL ampicillin. The protein was then induced with a final concentration of 0.1 mM IPTG (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), and the E. coli was cultured overnight at 16 °C. The bacterial pellets were sonicated well and centrifuged to collect the supernatant. The protein was purified from the supernatant with Ni-NTA resin (Fujifilm Wako Pure Chemical Corporation) according to the manufacturer’s protocol. The elution was dialyzed twice against 50 mL of phosphate-buffered saline (PBS) and the protein concentration was determined by Bradford method.

Cell cultures and cell fusion

The source and culture conditions of the myeloma cell line (SP2/0-Ag14 [RCB0209]) were the same as described in Akagi et al. (6). Anti-EGFP hybridoma (2H12) and anti-αTubulin hybridoma (3A1) were established in our laboratory by conventional method. BALB/c female mice were immunized intraperitoneally with an emulsion of improved Freund’s complete adjuvant and 100 μg His-EGFP protein. Then, 18 days later, the mice were intraperitoneally boosted with 100 μg His-EGFP protein in PBS containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM NaH₂PO₄ with a pH of 7.2. After 3 days of the final immunization, the mice were sacrificed, and the spleenocytes were collected in PBS. The procedures for cell fusion and purification of hybridoma were the same as those in the previous paper (6). The protocols for the animal experiments were reviewed and approved by the Ethics Committee on Animal Experiments of Yokohama National University.

**Antigen-mediated fluorescent labeling to hybridoma and flow cytometric sorting.**

Hybridomas were subjected to antigen-mediated fluorescent labeling. His-EGFP recombinant protein (2 nmol) was mixed with hybridoma in 1 mL RPMI 1640 medium supplemented with 10% newborn calf serum (Biowest, Nuaille, France), and the cells were incubated for 2 h at 37 °C in 5% CO₂. The cells were centrifuged at 12,000 rpm at 800 rpm for 3 min, washed three times, and finally resuspended in 500 μL RPMI 1640 medium. The hybridoma cell sorting was carried out as described previously (7).

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**Characterization of obtained mAbs**

For the ELISA assay, the protocol was essentially the same as described previously (9). For the screening antigen, it can be fluorescently-labeled screening peptide binds to mIgs expressed by an antibody-secreting hybridoma. Fluorescently-labeled hybridomas that are not expected to produce antigen-specific antibodies are then selected and cloned by FCM. When a peptide antigen is used as a screening antigen, it can be fluorescently labeled with PE-streptavidin to chemically synthesize a biotin terminally-labeled screening peptide. However, when biotin is chemically introduced into a recombinant protein expressed in E. coli, biotin is randomly introduced into the lysine residue of the amino acid sequence, which may change the epitope structure. To avoid structural alteration of the epitope, the target protein was fused with the fluorescent protein EGFP, and the fusion protein was used as a fluorescent tag (Fig. 1A). In a pilot experiment, the EGFP protein bound to a 2H12 anti-EGFP mAb-secreting hybridoma (Fig. 1B).

**Hybridoma labeling conditions using EGFP recombinant protein.**

To develop this system, the fluorescent labeling conditions were investigated using EGFP recombinant protein and a hybridoma (2H12) that secretes anti-EGFP monoclonal antibody. The labeling conditions for recombinant proteins were determined based on the peptide labeling conditions (Figs. S2 and S3). The results showed that the mode of fluorescence intensity distribution was saturated at 2 nmol when labeled with the screening recombinant protein for 2 h. Therefore, this condition was used for hybridoma labeling in the following experiments.

**Establishment of anti-EGFP mAbs using the rMIHS method.**

In this study, anti-EGFP mAbs were generated, and an FCM analysis was performed (Fig. 2). Unlabeled hybridomas exhibited fluorescence intensity indicated by the black line, whereas significantly-labeled 0.92% cells in the enlarged region of Fig. 2 were single-cell sorted on 96-well plates containing RPMI 1640 medium with 10% NBS + HAT. Then, the cells were cultured for 7 days, the culture supernatant was obtained, and the reactivity with the EGFP protein was examined by western blotting (Fig. S4). A summary of the comparison between the ELISA method and the rMIHS method is shown in Table 1. After performing the same procedure twice, 15.2% of the clones showed specific reactivity with the recombinant EGFP protein (Table 1). Similarly, unlabeled hybridomas were single-cell sorted by FCM on a 96-well plate containing 200 μL of the same
medium, cultured for 10 days, and the culture supernatants were examined by western blotting to determine the reactivity with the EGFP protein. As the result, 0.75% of the clones showed a single band, and the efficiency of obtaining positive clones by rMIHS was 20 times higher than the ELISA. In addition, as in the case of using peptide antigens, the isotypes of the clones obtained by the rMIHS method showed high probability (65 clones out of 72 clones) to get IgG subclasses (Fig. S5).

Evaluation of functional structure-specific mAbs obtained in this study Since the rMIHS method preferentially obtains antibodies that recognize the protein structure, the ELISA intensity and the fluorescent labeling intensity of the clones obtained by the ELISA method and the MIHS method were compared (Fig. 3). Plot patterns can be roughly divided into three groups A, B, and C. Group A is a group of clones in which the FCM fluorescence intensities were very low, although strong signals were obtained by ELISA, and most of them were obtained by the ELISA method. Group B contained clones with fluorescence intensities commensurate with the ELISA intensities, most of which were obtained by rMIHS. Lastly, group C is a group of clones with very high fluorescence intensities compared to ELISA intensities, and all of the clones were selected by rMIHS.

Immunoprecipitation experiments are usually analyzed by immobilizing mAbs on protein G carriers and binding them to proteins. However, in our experience, some IgG isotypes of mAbs did not bind to protein G. Therefore, biotin was introduced sequence-specifically with BirA into the recombinant EGFP protein fused with the BirA recognition sequence (9), the protein was fixed to streptavidin magnetic beads, and the hybridoma culture supernatants were applied to the beads. The mAbs bound to the protein were analyzed by western blotting using anti-mouse IgG-HRP. As with normal immunoprecipitation, this method identifies mAbs that recognize functional structures of the protein. As shown in Fig. 4, most of the examined mAbs in groups B and C were immunoprecipitated, whereas some of the ELISA-derived clones in group A were not immunoprecipitated.

A modified Capture ELISA was performed to investigate the effect of structural changes in EGFP on mAb reactivity. Since the denatured and biotinylated EGFP-BirHis protein could bind to streptavidin (Fig. S6), boiled and unboiled EGFP-BirHis are immobilized on a streptavidin-coated ELISA plate (Fig. 5A). SDS binds non-specifically to denatured proteins, thus reducing the
unwinding the protein structure. Therefore, when the biotinylated EGFP-BirHis protein was boiled, the effect on the boiling with and without SDS were compared. The culture supernatant of each clone was added to the well to check the reactivities of the antibodies with the coated proteins (Fig. 5B). EGFP quenches green fluorescence by boiling indicates that unboiled EGFP has a functional structure and the structure is denatured by boiling. In order to investigate the difference in the reactivity of mAb due to structural changes, 29 clones obtained by the rMIHS method and 22 clones obtained by the ELISA method were analyzed by the Capture ELISA method. As a result, the number of clones that responded only to EGFP with a functional structure and not to heat-denatured EGFP was 22 clones (75.9%) by the rMIHS method and 8 clones (36.4%) by the ELISA method. Fisher's exact test indicates that the rMIHS method can obtain antibodies that recognize functional structures with significantly higher efficiency than the ELISA method. On the other hand, the proportion of clones that recognized denatured EGFP was higher in the ELISA method than in the rMIHS method, although there was no significant difference.

**DISCUSSION**

In general, mAbs can be divided into two types: those that recognize linear epitopes of the primary structure of proteins and those that recognize conformational epitopes of secondary and tertiary structures (2). Monoclonal antibodies against many kinds of proteins have been established, but most of them bind to denatured antigens. Antibodies that bind to denatured antigens are not effective in the study of fully functional proteins or in drug development targeting fully functional proteins. Therefore, stereospecific monoclonal antibodies that specifically bind to target antigens are preferred for these types of applications (3).

The ELISA method is generally used for the primary screening of hybridomas. ELISA screening likely does not destroy the antigen structure completely during the immobilization on the plate, but the higher-order structure of the protein formed by the distal amino acid has a high probability of changing its structure. Therefore, when ELISA is used for hybridoma screening, it is difficult to obtain an antibody that specifically recognizes the higher-order structure of a protein.

The method for selecting stereospecific mAbs commonly uses mlg expressed on B cells and hybridoma cell membranes as screening tags and can be broadly divided into two depending on the screening timing (2). Tsumoto et al. (3) developed a stereospecific targeting (SST) method where myeloma cells selectively fuse with B cells that produce antigen-specific antibodies prior to the cell fusion. A recent study reported an optimized version of this method, which has improved the efficiency of obtaining hybridomas that produce the target antibody (11).

A method of screening clones that produce antigen-specific antibodies after cell fusion is an FCM-based methodology that enables the selection of a single cell from a bulk mixture of fused hybridoma cells. The method does not require special reagents, is

![FIG. 2. Representative FCM profile from the fusion mixture. The cells cultured after fusion between EGFP-primed B cells and the myeloma cells were examined by FCM. When compared to the FCM profile of the unlabeled fused cells (mode of fluorescence intensity indicated as M), 0.94% of the cells were in the positive area (more than 3M). The positive area is enlarged and colored gray.](image-url)

**TABLE 1. Summary of hybridoma generations of rMIHS and ELISA method.**

| Method      | Total number of clones analyzed | Western blot analysis | No. IgG clones |
|-------------|---------------------------------|-----------------------|----------------|
|             | EGFP expressed in *E. coli*     | EGFP expressed in *E. coli* |                |
| Conventional (N = 3) | 1200  | 43<sup>c</sup>  | 22              | 11          | 9 (0.75<sup>b</sup>) | 15 (33%) |
| rMIHS (N = 2)  | 375   | 54<sup>c</sup>  | 72<sup>a</sup>     | 60          | 57 (15.2<sup>c</sup>) | 65 (90%) |

*The experiments were performed twice by the MIHS method and three times by the ELISA method, and all the results are summarized. Conventional screening was performed in the order of ELISA, western blotting of *E. coli* expressed EGFP, and western blotting of EGFP expressed in eukaryotic cells. Screening for the rMIHS method started with a Western analysis of EGFP expressed in *E. coli*. N is the number of screens.

<sup>a</sup> The number of clones that were positive in the initial screening.

<sup>b</sup> The percentage of clones that specifically react with EGFP on the western blot is calculated as (number of clones that gives single band) / (total number of clones analyzed) × 100.

<sup>c</sup> The procedure of *E. coli* western blotting using antibodies from clones identified by rMIHS is not included in the screening procedure, but the number is provided for comparison between the two methods.
easy to set up, and can be applied in many different types of laboratories \((8,11-14)\). By advancing both methods (SST and MIHS), it will be possible to obtain a variety of monoclonal antibodies suitable for specific applications.

In the previous paper, we have developed the MIHS method using peptide antigens \((8)\). The method saves time and effort compared to traditional multi-microwell plate seeding and subcloning by limiting dilution \((2)\), avoiding the labor and time of a cumbersome repetitive screening process. In the previous reports comparing the fluorescence intensity of hybridomas measured by FCM to the colorimetric intensity measured by ELISA, there were clones showing strong fluorescence intensity even though the colorimetric intensity was weak. We expected that these clones might be structure recognition mAbs. Although it is potentially

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**FIG. 3.** Correlation of ELISA absorbances and fluorescence intensities. The vertical axis shows the ELISA absorbances (405 nm) of the antibodies from hybridoma clones generated by rMIHS. The horizontal axis shows the fluorescence intensities of the hybridomas measured by FCM. Each clone was plotted with one dot. Open circles indicate clones obtained by rMIHS, and closed triangles indicate those obtained by ELISA. The distribution of dots is divided into three groups according to the relationship between the fluorescent label measured by FCM and the emission intensity measured by ELISA.

**FIG. 4.** Immunoprecipitation and isotype determination of the clones produced by rMIHS. mAbs of M2B2 and M3F1 (rMIHS clones) and 14E5 and 1G4 (ELISA clones) did not immunoprecipitated, but other mAbs were immunoprecipitated. Note that most of the mAbs in groups B and C were immunoprecipitated. The lower figure indicates the presence of antibodies in solutions used in the culture supernatants and their isotopes.
difficult for short-chain peptides to form a structure, we predict that the antigenic peptides (427–441 aa region of mouse α-tubulin) used in previous reports correspond to α-helices (data not shown). Since the α-helix forms a secondary structure through the interaction between proximal amino acids, it was interpreted that some of the mAb made from this short-chain peptide recognizes the secondary structure.

In this paper, an rMIHS method was developed using a recombinant protein antigen expressed in E. coli and tested to confirm whether this method is suitable for obtaining a functional structure-recognizing antibody. In the rMIHS method, the screening antigen must be fluorescently labeled. Therefore, we obtained mAbs using EGFP recombinant protein as a fluorescent tag. Based on the results, the efficiency in obtaining mAbs and IgG antibodies and the reaction specificities of the mAbs showed the same effectiveness as the peptide MIHS method. Furthermore, a comparison of the labeling intensity by FCM and the colorimetric intensity by ELISA (Fig. 4), and Capture ELISA (Fig. 5) confirmed that many mAbs obtained by the rMIHS method are functional structure recognition antibodies.

Various methods have been used to identify antibodies that recognize the structure of proteins. These methods include immunoprecipitation, immunostaining, sandwich ELISA, Cell-ELISA (15), and so on. When preparing protein extracts for western blotting, there were clones with different reactivity with and without boiling (16). Clones were obtained that did not show reproducible results when western blotting was performed (data not shown). Therefore, modified Capture ELISA was used to compare the reactivity between the heat-denatured antigen and the antigen with functional structure. This method was highly reproducible and could reliably compare the reactivity of denatured/functional antigens with mAbs, indicating that the structure recognition antibody can be easily and accurately identified by rMIHS.

The rMIHS method for recombinant protein antigens developed in this study has the same advantages as the MIHS method (simple tedious screening and cloning steps, high throughput, and short duration of less than 40 days from the first immunization to the clone establishment). In addition, the rMIHS method has the important advantage of obtaining a high proportion of antibodies that specifically recognize functional structure of the target protein. This method would be highly useful for any laboratory with conventional hybridoma technology and an FCM.

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