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B-cell receptor-based multitargeting method for simultaneous production of novel multiple monoclonal antibodies

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Until now, various kinds of monoclonal antibodies have been raised against many antigens. Nevertheless, the production of these monoclonal antibodies was usually limited to only one antigen. If simultaneous generation of monoclonal antibodies against multiple antigens were available at one time, we could reduce not only laborious work, but also experimental animals. Here, we developed a multitargeting (MT) method that enables simultaneous production of monoclonal antibodies against multiple antigens on the basis of strict selection of sensitized B lymphocytes by the target antigens via B-cell receptors. After immunization using multiple antigens, monoclonal antibodies against four different antigens containing lower antigenic one were successfully generated only in one experiment. At maximum, more than 90 % of ELISA-positive wells to hybridoma-positive ones was obtained by this advanced technology. This must be attributed to strict selection of sensitized B lymphocytes by different antigens. In the MT method, sensitized B lymphocytes were selected by means of each desired antigen regardless of their antigenic differences. Selective fusion of B cell–myeloma cell complexes by electrical pulses was also of critical importance for efficient generation of hybridoma cells secreting desired monoclonal antibodies. This study strongly suggests that simultaneous production of novel monoclonal antibodies directed against multiple antigens of interest by the MT method can be feasible.

[**Key words:** B-cell receptor; Biotin/streptavidin binding; Hybridoma cell; Monoclonal antibody; Multitargeting]

Vertebrates have sophisticated immune systems that can protect against all kinds of infectious attacks by viruses and bacteria. Innate immunity is a fundamental system for inactivation of foreign antigens at an early stage, whereas acquired immunity represents antigen-specific immune responses that are activated against antigenic determinants in the targeted foreign pathogens. The acquired immunity consists of two major systems such as cell-mediated immunity and humoral immunity. In the latter immune response, B lymphocytes play pivotal roles in producing antibodies specific for various kinds of foreign antigens.

Monoclonal antibodies have been widely employed for diverse purposes, including basic sciences and medical applications. Recently, they have been utilized as a therapeutic medicine because of their high specificity, few side effects, and relatively longer blood clearance. The original method for generating monoclonal antibodies was reported by Köhler and Milstein in 1975 (1), where they used hemagglutinating virus of Japan (HVJ) to fuse B lymphocytes with myeloma cells to produce hybridoma cells secreting monoclonal antibodies. Later on, poly (ethylene glycol) (PEG) as well as HVJ became available (2).

Nonetheless, when HVJ and PEG were used for the fusion, myeloma cells were nonspecifically fused not only with the target antibody-producing B lymphocytes but also with other undesired B lymphocytes and splenic cells. Such undesirable fusion reduces the efficiency of fusion of a desired pair of cells, requiring much time and labor for screening and cloning of hybridoma cells secreting desired monoclonal antibodies.

Some time ago, our group developed a technology termed pulsed electric field (PEF) method (3–7), which also became known as a B-cell targeting (BCT) technique later (8–12).

This new technology consists of three critical steps. The first step is the selection of immunized B lymphocytes by means of a desired antigen based on B-cell receptors (BCRs). The second step is the formation of B cell–myeloma cell complexes by using specific and high affinity between biotin and streptavidin. The final step is selective fusion of B cell–myeloma cell complexes by electrical pulses to generate hybridoma cells secreting desirable monoclonal antibodies. This technology enabled us to generate desired monoclonal antibodies against target antigens with high efficiency and selectivity. Moreover, the BCT method has been effective not only for protein antigens but also for peptide antigens and low-molecular-weight compounds (8). The usefulness of the BCT method has also been demonstrated by other research groups (13–15). Each critical step of the BCT method was also successfully proved by immunofluorescence analysis (9). Even though the BCT method enabled selective production of monoclonal antibodies with high efficiency, only one antigen was available for one experiment. If we can generate monoclonal antibodies against multiple antigens at once, this method should be beneficial and practical for many applications.

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In the present study, we focused on simultaneous production of monoclonal antibodies against multiple antigens in one experiment. For this purpose, we focused on strict selection of sensitized B lymphocytes by means of each target antigen via BCRs. The critical point in this regard is that one B lymphocyte harbors one specific BCR against one epitope of a target antigen.

MATERIALS AND METHODS

Materials Bovine serum albumin (BSA), ovalbumin (OVA), rabbit serum albumin (RSA), human hemoglobin (hHb), and hen egg lysozyme (HEL) were purchased from Wako (Osaka, Japan). *N*-Hydroxysuccinimide (NHS)-biotin was acquired from Sigma-Aldrich (St. Louis, MO, USA), and horseradish peroxidase (HRP)-streptavidin conjugate was purchased from ZyMED Laboratories (South San Francisco, CA, USA). Streptavidin (StAv) type II, *N*,*N*-dimethylformamide (DMF), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were acquired from Wako. An anti-mouse IgG (H+L) antibody conjugated with HRP was purchased from BioSource International (Camarillo, CA, USA).

Mice BALB/c mice were acquired from CLEA Japan (Tokyo, Japan). Female mice at the age of 5–7 weeks were used for immunization.

Ethical conduct of research All experiments were conducted according to Mie University's guidelines for the care and treatment of experimental animals.

Cell culture PAI murine myeloma cells (16) were grown in the complete RPMI 1640 medium supplemented with 10% of fetal calf serum, 2 mM L-glutamine (Nissui Pharmaceutical Corporation, Tokyo, Japan), and 100 μg/mL kanamycin sulfate (Meiji Seika Corporation, Tokyo, Japan) at 37°C in a humidified atmosphere containing 5% of CO_2.

Production of biotinylated antigens One milligram of an antigen dissolved in phosphate-buffered saline (PBS) (1 mg/mL) was mixed with 20 μL of NHS-biotin (1 mg per 30 μL of DMF) and gently rotated at room temperature for 30 min. After that, 20 μL of 1 M glycine was added, and the mixture was rotated further at room temperature for another 30 min to block the NHS functional group in NHS-biotin. The biotinylated antigens were confirmed by SDS-PAGE and an enzyme-linked immunosorbent assay (ELISA).

Immunization BALB/c mice at 5–7 weeks of age were used for immunization. Each antigen was mixed with CFA at the ratio of 1:1 (v/v). Water-in-oil (w/o) emulsion containing the antigen was injected intraperitoneally. Two weeks later, each antigen in incomplete Freund's adjuvant (IFA) at the ratio of 1:1 (v/v) was employed for the second immunization. Immunization was continued up to five times at 2-week intervals for hHb and HEL, whereas sensitization was carried out up to three times at 4-week intervals for BSA, OVA, and RSA, as shown in Supplementary Table S1. Antibody production in the immunized mouse was evaluated by an ELISA method.

Preparation of spleen cells Spleen cells from an immunized mouse were aseptically prepared by a method reported previously (5,8).

Multitargeting method Spleen cells (5×10^7 to 1×10^8) containing B lymphocytes sensitized by five different antigens (hHb, HEL, BSA, OVA, and RSA) were suspended in 2.5 mL of DMEM containing 100 μg/mL kanamycin sulfate (DMEM$^+$). Twenty micrograms of each of the five biotinylated antigens (total 100 μg of biotinylated antigens) dissolved in another 2.5 mL of DMEM$^+$ as a cocktail were mixed with 2.5 mL of the above-mentioned splenocyte suspension, and the mixture was gently rotated for 2 h at 4°C. For selection of B lymphocytes with two different antigens, 40 μg of biotinylated antigens (20 μg bio-HEL and 20 μg bio-OVA) were dissolved in the aliquot (2.5 mL) of DMEM$^+$. After that, the cell suspension was centrifuged at 2000 rpm (800 $\times g$) for 5 min, and the pellet was resuspended in 10 mL of DMEM$^+$. After centrifugation at 800 $\times g$ for 5 min again, the spleen cell pellet was resuspended in 2.5 mL of DMEM$^+$. Another 2.5 mL of DMEM$^+$ containing 200 μg of streptavidin was added to the spleen cell suspension and carefully mixed by rotating for 1 h at 4°C. Finally, the spleen cell pellet after washing with DMEM$^+$ was resuspended in 5 mL of DMEM$^+$. At this step, B cell-antigen-biotin-streptavidin complexes were formed.

PAI murine myeloma cells (2×10^7 to 3×10^7) were collected by centrifugation at 800 rpm (130 $\times g$) for 5 min and resuspended in 10 mL of sterile PBS (PBS$^+$). After washing by PBS$^+$, the myeloma cell suspension in 5 mL PBS$^+$ was mixed with 5 mL of PBS$^+$ containing 10 μL of NHS-biotin (1 mg per 30 μL DMF), and the mixture was carefully rotated for 30 min at 37°C. Biotinylated myeloma cells were pelleted by centrifugation at 130 $\times g$ for 5 min and washed with 10 mL of DMEM$^+$.

A spleen cell suspension containing B cell-antigen-biotin-streptavidin complexes were mixed with biotinylated myeloma cells at a ratio of 10:1 to 1:1 (spleen cells:myeloma cells) and centrifuged at 1000 rpm (200 $\times g$) for 10 min and resuspended in 1 mL of DMEM$^+$. The cell mixture was gently rotated for 0.5–1.0 h at room temperature. At this step, B lymphocytes were combined with biotinylated myeloma cells through strong and specific interactions between biotin and streptavidin. The resulting B cell–myeloma cell complexes were resuspended in 1–2 mL of isotonic sucrose buffer composed of 0.25 M sucrose, 2 mM NaH_2PO_4/Na_2HPO_4 (pH 7.2), 0.1 mM $MgCl_2$, and 0.1 mM $CaCl_2$. Finally, the cell complexes were fused, as reported previously (6–8). Basic protocol for multitargeting (MT) technique was performed, according to Tomita et al. (8,9,17).

Flow chart of MT technique is shown in Fig. 1.

Cloning of hybridoma cells By using a limiting dilution method, hybridoma cells were diluted to 9, 3, 1 and 0.5 cells/well and incubated in complete RPMI medium with humidified 5%: 95% CO_2/air gas mixture at 37°C for a few weeks.

Screening for hybridoma cells After target antigens (10 μg/mL) were adsorbed on a 96-well plate overnight at 4°C, each well was blocked with 1% gelatin in PBS by incubating for 2 h at 37°C. Antiserum or supernatant from hybridoma cells was added as a primary antibody, and a 1/10,000 diluted goat anti-mouse IgG (H+L) antibody conjugated with horseradish peroxidase (HRP) was used as a secondary antibody. Finally, the absorbance in each well was measured at 490 nm using *o*-phenylenediamine as a substrate (5).

Determination of the biotinylation rate for antigens Hydroxyazobenzene-2′-carboxylic acid (HABA, 1.4 mg) was dissolved in 50 μL of DMSO and mixed with 1 mg avidin in 950 μL of PBS to prepare a HABA/avidin solution. The measurement was carried out by mixing 50 μL of the biotinylated antigen in PBS with 50 μL of the HABA/avidin solution. The biotinylation rate for each antigen was determined on the basis of the ratio of complexes between biotin and

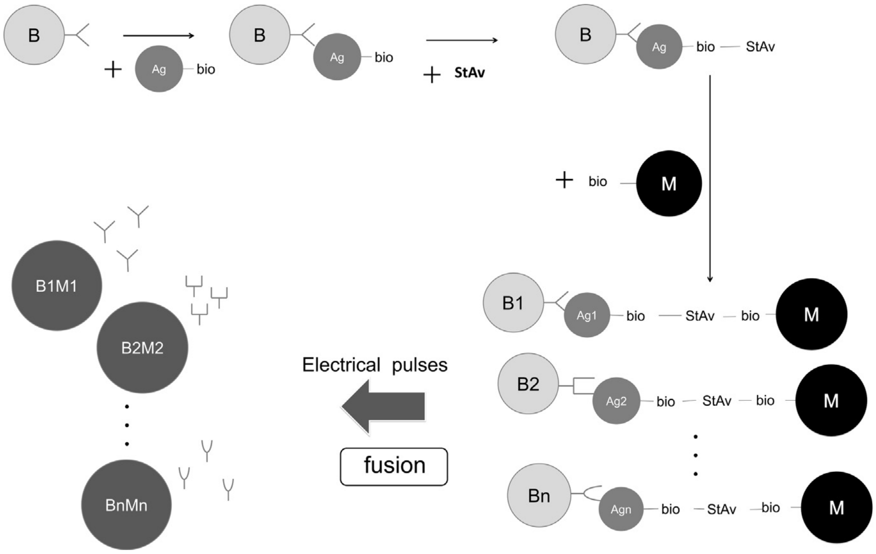

FIG. 1. Multitargeting (MT) method. B lymphocytes sensitized by multiple antigens were selected by means of each biotinylated antigen. All antigen-selected B lymphocytes were combined with myeloma cells via specific binding between biotin and streptavidin. All B cell–myeloma cell complexes were electrically fused to simultaneously produce hybridoma cells secreting each desired monoclonal antibody. B, B lymphocyte; Ag, antigen; M, myeloma cell; bio, biotin; BM, hybridoma cell; StAv, streptavidin.

avidin ($K_d \approx 10^{-15}$ M) and between HABA and avidin ($K_d \approx 10^{-6}$ M). A decrease in absorbance of HABA/avidin at 500 nm, after binding to the biotinylated antigens with avidin, was determined according to Green (18) and Livnah et al. (19). A calibration curve was constructed by means of different concentrations of D-biotin. The biotinylation rate for each antigen was calculated on the basis of Lys residues in each antigen by means of the calibration curve.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 14% slab gel under reducing conditions (20), and the separated proteins were stained with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA).

Immunoblot analysis The proteins separated by SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane at 20 V for 90 min, and soaked in 1% gelatin in PBS for 90 min at room temperature. After rinsing the PVDF membrane three times with PBS containing 0.05% of Triton X-100 (PBST), the membrane was incubated for 1 h with a primary antibody as antiserum or supernatant from hybridoma cells. After rinsing with PBST, the PVDF membrane was incubated for 1 h with a secondary antibody at 1/10,000 dilution of a goat anti-mouse IgG (H+L) antibody conjugated with HRP. After at least five washes with PBST, the proteins on the PVDF membrane were visualized by means of a Konica Immunostain HRP-1000 Kit (Seikagaku Kogyo, Tokyo, Japan).

Quantification of protein Protein concentration was determined according to a Bio-Rad protein assay system with BSA as a standard.

RESULTS AND DISCUSSION

Evaluation of biotinylated antigens Lys residues in five kinds of antigens such as hHb, HEL, BSA, OVA, and RSA were biotinylated by NHS-biotin and analyzed by SDS-PAGE. As a result, small, but noticeable shifts of each biotinylated antigen to higher molecular weights were detected (data not shown). The modified antigens showed relatively smeared bands indicating hetero-biotinylation. In addition, the biotinylated antigens except bio-HEL were found to be specifically bound to streptavidin (Fig. S1A). In the case of HEL, dilution of protein concentration from 1 mg/mL to 0.5 mg/mL in biotinylation brought about an increase in the amount of binding to streptavidin at least five fold (Fig. S1B). The antibody-binding ability of bio-BSA, bio-OVA, and bio-RSA was retained at almost the same level as before biotinylation, whereas that of bio-hHb and bio-HEL decreased after biotinylation (Fig. S1C).

Immunization with multiple antigens Intraperitoneal injections of multiple antigens were carried out for immunization. We first focused on possibility of immunization by multiple antigens including weakly antigenic proteins. We assumed that it might be difficult to immunize HEL due to a low molecular weight 14,307 Da (21–23), and also human Hb (hHb) because of its high homology (85%) to mouse Hb (24). To enhance sensitization for these two antigens with weaker antigenic properties, a dose of antigens as well as the number of immunization times was increased, as shown in Table S1. Another possible adverse effect on immunization with less antigenic proteins may be the interference by higher antigenic proteins for sensitizing B lymphocytes.

Effects of multiple antigens on immunization Next, we studied the interference by multiple antigens with the immunization. Production of antibodies in the serum against each antigen was evaluated in the presence of other antigens. To confirm the result, we selected a less antigenic protein such as HEL. The results revealed that the increase in the titer of the antibody against HEL in serum showed only a small difference between HEL alone and HEL in the presence of the other four antigens (Fig. 2A). Immunization with a single HEL antigen yielded a maximal titer in serum after the fourth booster, which was one-shot earlier than that obtained in the presence of multiple antigens. Fundamentally no significant influence was observed in the presence of other antigens on the immunization with a single antigen, even though a less antigenic protein (HEL) was employed. Consequently, B lymphocytes were successfully sensitized by less antigenic HEL in the presence of several more antigenic antigens. Immunization with multiple antigens proved to have a negligible effect on the sensitization for each desired antigen.

Strict selection of B lymphocytes by means of the target antigens After a mouse was immunized with five different

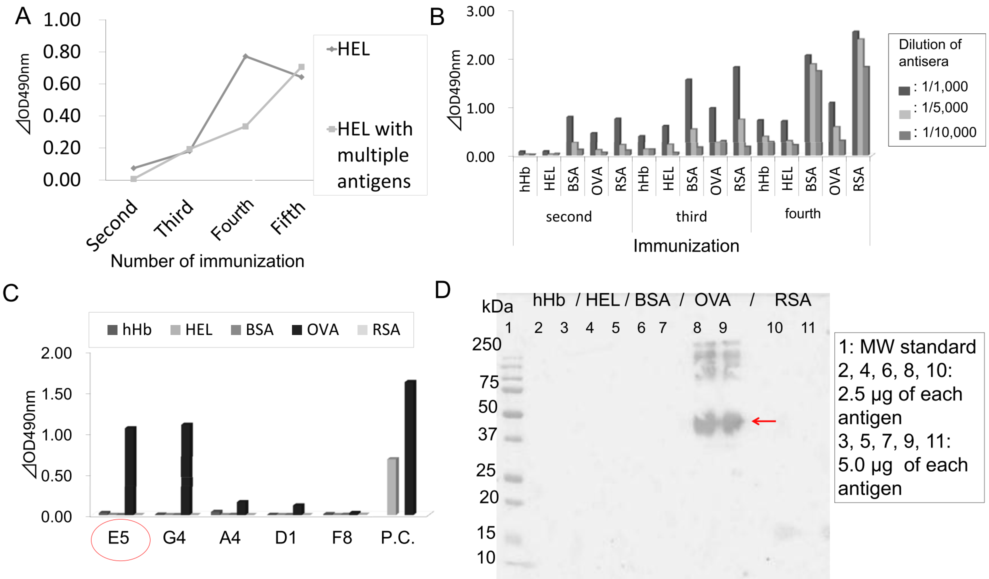

FIG. 2. Selective production of monoclonal antibodies against aimed antigens by the multitargeting (MT) technique. (A) The titer of the antibody in 1/1000-diluted serum for HEL was evaluated after immunization with HEL alone or HEL in the presence of hHb, BSA, OVA, and RSA. A HEL-coated 96-well plate was used in an ELISA assay. Diluted antisera from immunized mice served as primary antibodies. (B) The titer of antibodies in serum samples after immunization with five different antigens were determined by an ELISA method. Diluted antiserum for each antigen with 1/1000, 1/5000, and 1/10,000 was used as a primary antibody. (C) Monoclonal antibodies in the culture supernatant of hybridoma cells were determined. Hybridoma cells were obtained after selecting sensitized B lymphocytes by means of bio-HEL and bio-OVA, followed by fusion with myeloma cells based on the MT method. As a positive control, serum samples against HEL and OVA were used. (D) Specificity for monoclonal antibody produced by hybridoma E5 was evaluated by western blotting analysis. A total of 2.5 and 5.0 μg of each of the following: hHb, HEL, BSA, OVA, and RSA were separated by SDS-PAGE and transferred to a PVDF membrane. The culture supernatant from hybridoma E5 served as a primary antibody.

antigens such as hHb, HEL, OVA, BSA, and RSA, the sensitized B lymphocytes were selected by means of two of their specific antigens with bio-HEL and bio-OVA and verified whether the selection of B lymphocytes via the target antigen was controlled strictly or not. The reason why these two antigens were selected is that HEL has relatively weak antigenic properties, whereas OVA has rather stronger antigenic properties. As shown in Fig. 2B, a sufficient titer of antibodies in serum samples against each of the three antigens BSA, OVA, and RSA was recognized after the fourth immunization. Nonetheless, less antigenic hHb and HEL showed a relatively lower titer, showing less than 1.0 at ΔOD_{490} with 1/1000 diluted serum.

Consequently, sensitized B lymphocytes were selected by bio-HEL and bio-OVA based on B-cell receptors and fused with myeloma cells, according to the MT technique, as shown in Fig. 1. As a result, successful generation of monoclonal antibodies against OVA was observed (Fig. 2C). The monoclonal antibody obtained from hybridoma E5 showed specific binding to OVA without any cross-reactivity with other antigens according to Western blot analysis (Fig. 2D). This finding suggests that the selection of sensitized B lymphocytes by means of the target antigen though B-cell receptors is strict and precise. Even though multiple antigens were employed for immunization, the desired sensitized B lymphocytes were successfully selected by means of the corresponding antigen in the MT method. Here we could prove the fact that bio-OVA successfully selected the sensitized aimed B lymphocytes among various kinds of sensitized B lymphocytes immunized by five different antigens. However, we could not generate monoclonal antibodies against HEL after selecting B lymphocytes by bio-HEL.

Concerning the immunization for B lymphocytes, four-times sensitization for HEL seemed to be insufficient for selecting sensitized B lymphocytes (Fig. 2B). On the contrary, the titer in 1/1000-diluted serum after the fifth sensitization was more than 1.5 on day 5 after the final immunization, as shown in Fig. 3A. This discrepancy could be attributed to the number of activated B lymphocytes which must be available for selection by means of the bio-HEL. It seems that a sufficient number of sensitized targeted B lymphocytes against HEL were generated 5 day after the fifth sensitization, where the number of produced plasma cells from the memory B lymphocytes may also be increased. The decreased antibody-binding ability of bio-HEL, as shown Fig. S1C, would result in lower production of hybridoma cells secreting desired monoclonal antibodies. This is because the decreased binding ability of bio-HEL toward the antibody means less effective selection of sensitized B lymphocytes by means of bio-HEL using BCRs. We assume that the same reasoning would be applied to bio-hHb. However, we verified effective production of sensitized B lymphocytes for HEL (Table 1).

We are sure that this method enables selective production of non-cross-active monoclonal antibody against each antigen, because each of aimed sensitized B lymphocytes was pre-selected by each of the corresponding antigens before generating hybridoma cells secreting desired monoclonal antibody.

The best time for selecting sensitized B cells after the final immunization It is generally known that splenocytes are fused with myeloma cells to generate hybridoma cells with three days after the final immunization (1,2). We examined the best and most suitable time for B-cell selection after the final immunization because the selection of sensitized B lymphocytes by means of each antigen of interest is a critically important step of this MT method. Expression of antigen receptors on the sensitized B lymphocytes may determine the efficiency of selecting the immunized B lymphocytes. Fig. 3A shows that a sufficient amount of antibodies to each antigen in serum samples except hHb and HEL was observed on day 3–10 after the final booster. In the MT method, sensitized B lymphocytes were selected by

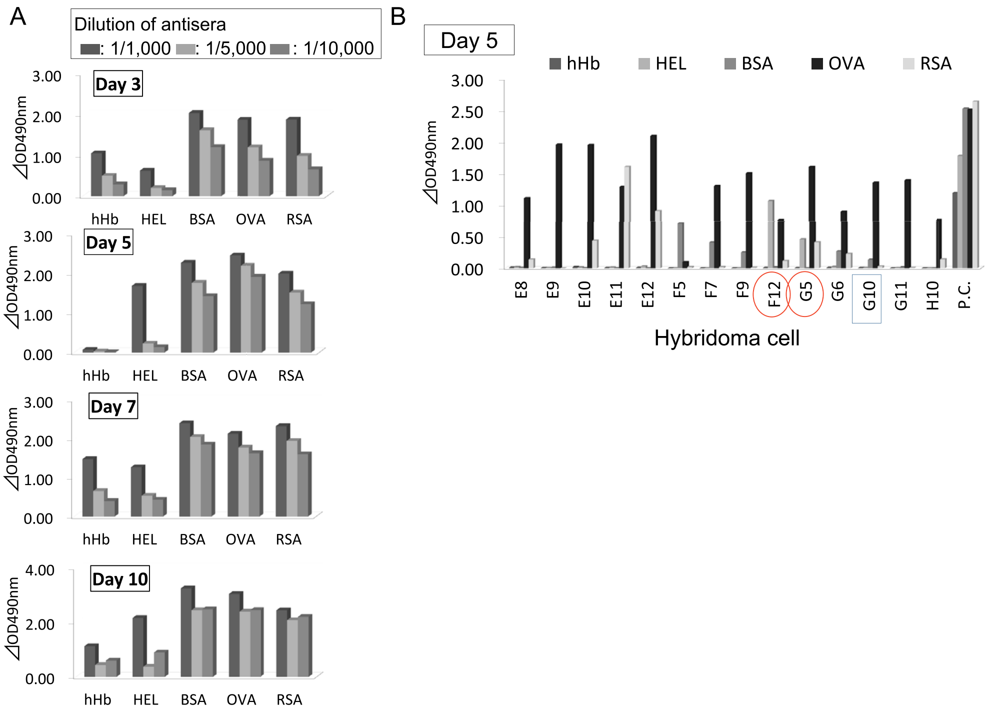

FIG. 3. Determination of ELISA-positive hybridoma cells fused on day 5 on the basis of the MT method. (A) The titer of antibodies in serum samples on days 3–10 after the final immunization. The titer of antibodies in serum samples after immunization with 5 antigens was compared on day 3, 5, 7 or 10 after the final booster. A diluted antiserum against each antigen with 1/1000, 1/5000, and 1/10,000 was used as a primary antibody. (B) Representative hybridoma cells secreting monoclonal antibodies. Sensitized B lymphocytes were fused with myeloma cells on day 5 after the final immunization on the basis of the MT technique. Antibodies secreted into the supernatant by hybridoma cells were validated against five antigens.

TABLE 1. Comparison of fusion efficiency on day 3, 5, 7 or 10 after the final immunization.

Antigens for selecting B cells	Electrical pulses for fusion[a] (kV/cm)	Number of wells	Fusion[b] (day)	Number of hybridoma positive wells	Number of ELISA-positive wells (%)[c]				
					hHb	HEL	BSA	OVA	RSA
Biotinylated five antigens[d]	2.0	96	3	15	0	0	0	15 (100)	6 (40.0)
	2.5	96	3	16	0	0	0	7 (43.8)	10 (62.5)
	2.0	96	5	3	0	0	0	2 (66.7)	1 (33.3)
	2.5	96	5	23	0	4 (17.4)	5 (21.7)	21 (91.3)	9 (39.1)
	2.0	96	10	3	0	0	0	0	0
	2.5	96	10	4	0	0	0	1 (25.0)	0

[a] Hybridoma cells were generated by fusing sensitized B-cell and myeloma cell complexes at 2.0 kV/cm or 2.5 kV/cm of DC pulse with square wave for 10 μs, repeated four times at 1-s intervals.

[b] Fused day after the final immunization.

[c] $\frac{\text{Number of ELISA-positive wells}}{\text{Number of hybridoma-positive wells}} \times 100$

[d] bio-hHb, bio-HEL, bio-BSA, bio-OVA and bio-RSA.

means of each biotinylated antigen on day 3, 5, 7 or 10, and fused with myeloma cells. For this purpose, we used separate mice prepared for fusion on days 3, 5, 7 and 10. It should be noted that we here adopted the cocktail of the five biotinylated antigens in the experiment of selection of sensitized B lymphocytes.

As a result, the fusion on days 3 and 5 after the final immunization revealed much more efficient production of monoclonal antibodies against multiple antigens, as compared with those obtained on days 7 (data not shown) and 10 (Table 1). Especially, when antigen-selected B lymphocytes were fused with myeloma cells 3 or 5 days after the final sensitization, 90–100 % of ELISA-positive wells to hybridoma-positive wells was maximally recognized (Table 1). Monoclonal antibodies directed against even less antigenic HEL were detected as hybridoma cells, F12 and G5 (Fig. 3B). In the present study, although the titer of antibodies in serum samples increased with time, after the final immunization, the production of hybridoma cells decreased. This result may be attributed to the mechanism of humoral immunity. After repeated stimulation with the antigen, memory B cells differentiate into more matured memory B cells harboring high-affinity antigen receptors and at the same time, some of them become plasma cells that can secrete antibodies (25). According to the present observations, the fusion in 3 or 5 days after the last immunization indicated the most appropriate time for selecting sensitized B lymphocytes based on BCRs, resulting in enhanced fusion efficiency and the increased number of hybridoma cells producing the desired monoclonal antibodies. In contrast, on days 7 or 10 after the final immunization, although antibody titers in serum samples increased, a decreased number of hybridoma cells were obtained. This could be explained as follows: many plasma cells were generated in 7 or 10 days after the final sensitization. Since plasma cells harbor no BCRs, only a limited number of sensitized B lymphocytes remained available to be selected by the antigens. This means the number of sensitized B lymphocytes may reach the maximum on days 3 and 5 after the last booster, whereas plasma cells generated from memory B lymphocytes increased in number on days 7 and 10.

In consequence, we could succeed in simultaneous production of monoclonal antibodies against four different antigens in one experiment, as shown in Table 1. This finding strongly indicates that the selection of sensitized B lymphocytes by the target antigens must be strict and more appropriate and effective for selecting sensitized B lymphocytes based on BCRs on day 3 or 5 after the final immunization.

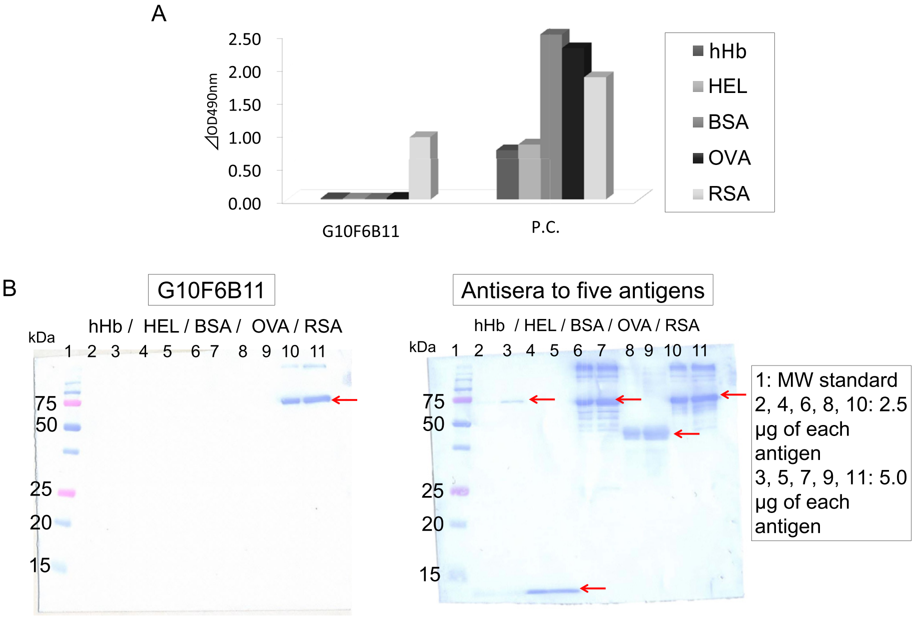

FIG. 4. Cloning of hybridoma cells by the limiting dilution method. A hybridoma cell line, G10F6B11, was obtained by cloning hybridoma cells G10 obtained in Fig. 3B by the limiting dilution method. Specificity for the monoclonal antibody produced by cloned hybridoma G10F6CB11 cells was determined by an ELISA and by western blotting.

TABLE 2. The biotinylation rate for each antigen.

		hHb[a]	HEL[a]	BSA[a]	OVA	RSA[a]
Molecular weight		64500	14300	69000	45000	68900
A	Lys residues (+N terminus)	46	7	60	21	59
B[b]	Biotinylation (nmol)	0.41	0.46	0.48	0.35	0.71
C	Total protein (nmol)	0.026	0.11	0.023	0.039	0.031
D[c]	Rate of biotinylation (%)	34.3	59.7	34.8	42.7	38.8
E	Possibly sterically hindered Lys residues	14	2	24	10	28
F[d]	Possible biotinylation sites (Lys residues)	32	5	36	11	31
G[e]	Assumed biotinylation sites (%)	69.6	71.4	60.0	52.4	52.5
H[f]	Possible rate of biotinylation (%)	49.3	83.6	58.0	81.6	73.9

[a] Average of two separate experiments.

[b] Determined by HABA method.

[c] $\mathrm{D} = \frac{B}{A \times C} \times 100.$

[d] $\mathrm{F} = A - E.$

[e] $\mathrm{G} = \frac{A - E}{A} \times 100.$

[f] $\mathrm{H} = \frac{B}{(A - E) \times C} \times 100.$

After repeated cloning of hybridoma cell line G10 (Fig. 3B) by a limiting dilution method, we were able to obtain the cloned hybridoma cell termed G10F6B11. To our surprise, monoclonal antibody produced by G10F6B11 showed strict specificity for only RSA without cross-reacting with the other four antigens, as shown in Fig. 4A and B, though we here tried to select hybridoma G10 secreting monoclonal antibody mainly against OVA but very little against to RSA (Fig. 3B).

In this study, absorbance value exceeding 1.0 just indicates whether sufficient sensitization and strong interaction between antibody and antigen are obtained or not. It does not seem to distort the main point for simultaneous production of novel monoclonal antibodies directed against multiple antigens by the multitargeting method. We here succeeded in cloning G10F6B11 that can secrete monoclonal antibody specific for RSA; of course, there are some detailed causes to be unveiled under the mechanism of the multitargeting selection.

The biotinylation rate for each antigen Although BSA is a strong antigenic protein, the rate for production of hybridoma cells against BSA was lower than that obtained against OVA and RSA in Table 1. To address this question, we determined the biotinylation rate for each antigen by a HABA method (26–31). Table 2 shows the percentages of biotinylation rate for BSA, OVA, and RSA, which indicated 34.8%, 42.7%, and 38.8% of the total Lys residues, respectively. However, this discrepancy may not cause the limited binding of biotinylated BSA to streptavidin, resulting in smaller formation of B cell-myeloma cell complexes to generate hybridoma cells secreting monoclonal antibodies.

Generally, the Lys residue is a target amino acid for biotinylation by NHS-biotin, where ε-NH_2 in the Lys residue nucleophilically attacks C=O in NHS to covalently tether a biotin to a protein. In this study, we hypothesized that lysine residues located near and adjacent to 2 or 3 amino acid residues is not available for biotinylation due to steric hindrance. Based on this assumption, the number of possible biotinylation sites (Lys residues) is 32 for hHb, 5 for HEL, 36 for BSA, 11 for OVA and 31 for RSA, as indicated by F in Table 2. Thus, a possible rate for biotinylation was estimated at 49.3% for hHb, 83.6% for HEL, 58.0% for BSA, 81.6% for OVA, and 73.9% for RSA, as shown by H in Table 2. These biotinylation rates may be subdivided into three groups. One is for RSA, OVA, and HEL whose rates are more than 70%. Another one is for BSA harboring approximately 60% of biotinylation. The third one is for hHb which contains less than 50%. This discrepancy could be well correlated with the present results. The relatively lower biotinylation rate for BSA, which is also shown in Fig. S1A, indicated substantially lower production of ELISA-positive hybridoma cells. In addition, the lower rate of biotinylation of hHb as well as its lower antigenicity, resulted in the absence of generation of ELISA-positive hybridoma cells.

In this study, we have just succeeded in selective production of four kinds of monoclonal antibodies against HEL, BSA, OVA and RSA in one experiment. However, this result strongly supports the simultaneous production of monoclonal antibodies by only one experiment, although we failed to generate monoclonal antibody against hHb, due to its lower biotinylation rate (49.3%), as shown in Table 2. The lower rate of biotinylation could cause poor formation of B cell and myeloma cell complexes by the specific and strong interaction between streptavidin and biotin (bio-Ag) for generating hybridoma cells secreting monoclonal antibody to hHb.

As a consequence, this study suggests the importance of strict selection of B lymphocytes by means of a desired antigen to simultaneously generate several monoclonal antibodies with high efficiency. We can produce monoclonal antibodies directed against even less antigenic proteins, regardless of the presence of more antigenic proteins. This result could be based on strict selection of target sensitized B lymphocytes via BCRs. We can conclude that the MT technique enables efficient production of monoclonal antibodies against various antigens robustly and simultaneously, even though less antigenic antigens are included. In addition, the rate of biotinylation may affect the selection of sensitized B lymphocytes and the formation of complexes of B lymphocytes with myeloma cells via streptavidin.

This study shows definitively that simultaneous generation of multiple monoclonal antibodies against each desired antigen in one experiment can be feasible. Strict selection of sensitized B lymphocytes by means of the target antigens via B-cell receptors is of critical importance. Here, we proved that specific monoclonal antibodies can be generated more effectively and robustly by the MT method.

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