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TECHNICAL NOTE

A modified hybridoma technique for production of monoclonal antibodies having desired isotypes

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Abstract In the present study, we describe a modified hybridoma technique for production of monoclonal antibodies (mAbs) having a desired isotype. Mice were immunized with the antigen of interest. After having reached a high antibody titer, cells expressing IgM or IgG molecules were isolated from spleen cells of the immunized mice using a Magnetic Cell Sorting System. The isolated cells were fused with myeloma cells using the conventional fusion protocol. With the isolated IgM^+ spleen cells, more than 75% (85 ± 7%; means ± SD) were IgM producing cells and a large number of IgM mAbs specific to the protein of interest were obtained. With the isolated IgG^+ spleen cells, 41 ± 40% of the generated hybridomas produced IgG antibody and no IgM producing hybridoma was generated. A large number of IgG mAbs specific to the protein of interest could be produced. The results indicate that the generated hybridomas produce corresponding antibody isotypes as expressed on the surface of their starting cells. The technique that we have developed will be very useful for production of desired mAbs having a specific isotype.

Keywords Hybridoma · Monoclonal antibody · Antibody isotype · Magnetic cell sorting

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Introduction

Following the discovery of hybridoma technology by Köhler and Milstein (1975), an assortment of monoclonal antibodies (mAbs) have been produced and have become important reagents used in biomedical research, in diagnosis and treatment of various diseases. In the conventional hybridoma technique, a mouse is immunized with the antigen of interest. After a high antibody titer has been reached, spleen cells of the immunized mouse are fused with myeloma cells to generate hybridomas that produce the mAbs of interest. This technique is generally familiar and widely available in many laboratories throughout the world. However, one obstacle to mAb production is that the obtained mAbs may have undecided isotypes.

There are several differences between IgG and IgM antibodies (Isaacs 2009; Janeway et al. 1999;

Lund et al. 1991). The molecular weights of the two molecules are different. IgG is a monomeric molecule while IgM is a pentamer. Under optimum conditions the combining sites of the IgG molecule span approximately 140 Å whereas the IgM molecule spans about 300 Å. The serum half lives of the two molecules are also different. IgG and IgM antibodies bind to different Fc receptors expressed on cell surfaces. In addition, different antibody isotypes play different roles in activation of the complement system. Due to these differences, a specific isotype of the mAb may be required for a specific purpose. For instance, in red blood cell agglutination testing, the IgM antibody is always required (Ernst et al. 1999). Red blood cells contain high zeta potential on their surface, which prevents them from being agglutinated by short-length IgG mAb. The polymeric IgM molecules are able to span distances between red blood cells in a manner that cannot be achieved by the IgG molecules. Therefore, almost all antibodies used in red cell grouping are of the IgM isotype. The hybridoma technique has been successfully applied to produce mAbs to red blood cell antigens for use in direct agglutination tests (Sonneborn et al. 1990; Sonneborn and Ernst 1987; Voak et al. 1981). However, in some cases, especially for protein antigens, production of IgM mAbs to red blood cell surface antigens was not achieved (Ernst et al. 1999; Schmitz et al. 1996) and most of the obtained mAbs were of the unexploited IgG isotype. In some other applications, the IgG mAbs are preferred. IgG mAb has been demonstrated to have higher affinity than IgM. In addition, purification of IgG mAbs is simpler than for the IgM isotype. However, in the conventional hybridoma technique, instead of obtaining the intended IgG mAbs, some mAbs of IgM isotype are always undecided obtained (Chiampanichayakul et al. 2006; Khunkaewla et al. 2007; our unpublished observations).

To overcome the problem of obtaining of unexpected isotype mAbs, in the present study we have modified the hybridoma technique for direct production of IgG or IgM mAbs against a desired antigen. Cells expressing IgG or IgM molecules were isolated from spleen cells of the immunized mice. The isolated cells were fused with myeloma cells using the standard hybridoma procedure. Using this approach, hybridomas producing IgG or IgM antibodies were obtained directly.

Materials and methods

Mouse immunization

Hemoglobin F (HbF) was purified from hemolysates of normal umbilical cord blood by DEAE Sepharose chromatography (Tayapiwatana et al. 2009). Briefly, the hemolysates were dialyzed against the binding buffer (Tris-HCl–KCN (THK) pH 9.0) for overnight. The C10/10 column containing DEAE Sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) was equilibrated with THK buffer pH 9.0. Afterward the hemolysate was applied to the column. The pH gradient separation for HbF was performed by increasing the concentration of eluting buffer (THK buffer pH 6.5) from 15 to 100% at a rate of 1% in every 5 min. The Hb fractions were collected by AKTA prime fraction collector (GE Healthcare Bio-Sciences). The obtained HbF was concentrated and the concentration of Hb was measured by cyanmethaemoglobinometry. The HbF obtained was checked for their purity by cellulose acetate electrophoresis.

For mice immunizations, purified HbF was mixed with complete Freud's adjuvant (Sigma–Aldrich, St. Louis, MO, USA) and immunized into four 6-weeks-old BALB/c mice (100 μg per dose). The immunizations were repeated with the same antigen using incomplete Freud's adjuvant (Sigma–Aldrich) at 2-week intervals, for two more immunizations. Blood samples were collected before immunization and at 2 weeks after the third immunization by tail bleeding. Mouse sera were determined for anti-HbF antibodies by indirect ELISA.

Immunofluorescent analysis

For enumeration of IgG and IgM expressing cells, spleen cells (1×10^7 cells/mL) were pre-incubated with 10% human AB serum at 4 °C for 30 min to block nonspecific Fc-receptor-mediated binding of antibody. The 5×10^5 blocked cells were then incubated for 30 min at 4 °C with Phycoerythrin (PE)-conjugated sheep $F(ab')_2$ anti-mouse IgG or IgM antibodies (Chemicon, Australia Pty Ltd, Victoria, Australia). Cells were then washed 3 times with PBS containing 1% BSA and analyzed for membrane fluorescence using a flow cytometer (FACSCalibur, Becton Dickinson Sunnyvale, CA).

Isolation of IgM and IgG expressing cells

When a sufficient antibody titer (>1:32,000) was detected, the immunized mice were sacrificed. IgM and IgG surface expressing cells were isolated from the immunized mice spleen cells by using a Magnetic Cell Sorting System (MACS) (Miltenyi Biotec, Bergish Gladbach, Germany). Briefly, spleen cells were mixed with rat anti-mouse IgM MicroBeads (Miltenyi Biotec) as described in manufacturer's instruction (1×10^7 spleen cells : 20 μL anti-mouse IgM MicroBeads) and incubated for 30 min on ice. Cells were washed twice with PBS containing 2 mM EDTA and 0.5% BSA (2 mM EDTA-0.5% BSA-PBS) by centrifugation. The cell pellet was resuspended in 500 μL 2 mM EDTA-0.5% BSA-PBS. The cell suspension was then applied onto the LD column and separated by MidiMACS Separator (Miltenyi Biotec) according to the manufacturer's instruction. The IgM surface expressing cells (IgM^+ cells) were magnetically isolated and contained in the positive fraction. The negative fraction of spleen cells was collected and labeled with goat anti-mouse IgG MicroBeads (Miltenyi Biotec) (1×10^7 spleen cells: 20 μL anti-mouse IgG MicroBeads) and the isolation performed as described above. The IgG surface expressing cells (IgG^+ cells) were collected. The final negative fraction, the non-IgM and IgG surface expressing cells (IgM^-/IgG^- cells), was also collected.

Modified hybridoma technique

The isolated IgM^+ cell, IgG^+ cell and IgM^-/IgG^- cell fractions were fused with myeloma cells (P3-X63Ag8.653) by a standard hybridoma technique. Briefly, the isolated cells were fused with myeloma cells at the cell ratio of 1:1 using 50% PEG (Sigma–Aldrich). After cell fusion, cells were resuspended in HAT selective medium (Sigma–Aldrich) to obtain a spleen cell concentration of 3×10^5 cells/mL and 100 μL of the cell suspension were seeded into each well of 96-well plates and cultivated at 37 °C in a 5% CO_2 incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA). After 5 days of cultivation, 150 μL of HT medium (Sigma–Aldrich) were added into each well. The plates were incubated at 37 °C in a 5% CO_2 incubator for hybridoma generation. The generated hybridomas were monitored by an inverted light microscope (Olympus, Tokyo, Japan).

Antibody isotyping

The isotypes of antibodies produced by the generated hybridomas were determined by indirect ELISA. Briefly, 50 μL rabbit anti-mouse immunoglobulins (10 μg/mL) (Dako, Glostrup, Denmark) were coated on a 96-well ELISA plate (Corning Incorporated, Horseheads, NY) using carbonate/bicarbonate coating buffer pH 9.6. The plate was blocked with PBS containing BSA (2% BSA-PBS). Hybridoma culture supernatants were added to each well and incubated at 37 °C for 1 h. The plate was washed 3 times with PBS containing 0.05% Tween 20 and 50 μL horseradish peroxidase conjugated goat anti-mouse IgM or IgG antibody (dilution 1:10,000) (Sigma–Aldrich) was added to each well and incubated at 37 °C for 1 h. Thereafter, 50 μL of tetramethylbenzidine (TMB) substrate (Zymed, South San Francisco, CA) was added. The reaction was stopped by adding 50 μL of 1 N HCl and the absorbance was measured at 450 nm by an ELISA reader (Tecan Austria Gmbh., Grodlg, Austria).

ELISA for anti-HbF antibody

Antibodies against HbF in mouse sera and in hybridoma culture supernatants were determined by indirect ELISA. Briefly, purified HbF (20 μg/mL) was coated on an ELISA plate. Then, the plate was blocked with 2% BSA-PBS. Mouse sera were diluted to various dilutions with PBS containing 0.05% Tween 20. The diluted sera or culture supernatants were added to each well and incubated at 37 °C for 1 h. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody at a dilution of 1:2,000 (DAKO) was added to each well and incubated at 37 °C for 1 h. Thereafter, the plate was washed and TMB substrate was added. The reaction was stopped and the absorbance was measured at 450 nm.

Results and discussion

Using the standard hybridoma technique (Köhler and Milstein 1975), mAbs specific to proteins of interest can be easily produced in laboratories. However, it is not always straightforward to obtain mAbs that have a specific isotype. This uncertainty is a drawback of some mAb applications, as different antibody isotypes

have different properties and utilizations (Isaacs 2009; Janeway et al. 1999; Lund et al. 1991). To overcome the uncertainty of obtaining a desired isotype mAb, in the present study, we modified the conventional hybridoma technique for generation of mAbs which have a specific isotype.

In this study, production of mAb to a protein antigen, HbF, was used as the study model. Mice were immunized three times with purified HbF. After the third immunizations, a suitably high anti-HbF antibody titer (titer more than $1 > 1{:}32{,}000$) could be detected in all immunized mice. Repeated antigen immunizations induce increased antibody production, affinity mutation and heavy chain class switching (Isaacs 2009; Lanzavecchia and Sallusto 2009; Abbas and Lichtman 2007). The appearance of a high antibody titer to the immunized antigen indicated that the antigen specific B cells were activated and differentiated into effector cells that actively produce antibodies of different isotypes.

Spleen cells were then segregated from the immunized mice. In the first mice, un-fractionated spleen cells were fused with myeloma cells using standard hybridoma technique. From the rest of the immunized mice, according to surface immunoglobulin expression, B cells expressing IgM and IgG were isolated from the spleen cells using the MACS system. The isolated cell fractions were then fused with myeloma cells for generation of hybridomas. By this approach, which is different from the standard hybridoma technique, the IgG and IgM expressing cells were isolated prior to the cell fusion. The isolated cells were then used as fusion partners for generation of hybridomas.

In this study, by using of un-fractionated spleen cells, after fusion, cells were seeded into a total of 960 wells. After cultivation in HAT selective medium, 359 wells (37%) of the seeded wells contained hybridomas (Table 1). All 359 hybridoma culture supernatants were screened for their antibody isotypes. Eighty percentage of the tested hybridomas produced IgM isotype antibody and 11% produced IgG isotype antibody (Table 1). Four percentage of the tested hybridomas were non-IgG or IgM producing cells and 6% produced both IgM and IgG isotypes (Table 1). Wells containing both IgM and IgG isotypes can be presumed to have multi-hybridoma clones which produce different antibody isotypes. The anti-HbF activity of the obtained hybridoma culture supernatants was also determined by ELISA. Seven percentage of the generated hybridomas were positive to HbF. Among of these, 5% were IgM and 2% were IgG isotype. We compared the obtained results to our previous un-published observation using Antigen 85, a *Mycobacterium tuberculosis* secreted protein, as the immunogen. Using Antigen 85 immunization, the percentages of hybridomas producing IgM and IgG were 26 and 43, respectively. Comparison between HbF and Antigen 85 immunizations suggested that different antigens induced different antibody isotype responses (Yamashita et al. 2002; Ernst et al. 1999; Schmitz et al. 1996; Yamada et al. 1993). The HbF, immunogen used in this study, induced a preference for the IgM isotype.

Un-fractionated spleen cells of the HbF immunized mouse were also stained for IgM and IgG expression and analyzed by flow cytometry. Within the spleen cells, percentages of IgM and IgG expressing cells

Table 1 Antibody isotypes produced by the hybridomas generated from un-fractionated spleen cells

Hybridomas obtained	Number of hybridoma containing wells in total 960 seeded wells[a]	% Of total hybridoma containing wells[b]
Total hybridomas	359	–
Hybridoma producing IgM antibody	287	80
Hybridoma producing IgG antibody	38	11
Hybridoma producing IgM+IgG antibody	20	6
Hybridoma producing none antibody	14	4

Spleen cells from HbF immunized mouse were fused with myeloma cells using standard hybridoma technique. After fusion, cells were seeded into 960 wells

[a] Number of hybridoma containing wells was determined. Culture supernatants were taken from the hybridoma containing wells and determined for antibody isotypes by ELISA

[b] % Of hybridoma producing IgG and IgM antibody was calculated from the total hybridomas obtained

were 38 and 29%, respectively. These results suggested that both IgM and IgG positive B cells existed in the segregated spleen cells. Some of them might produce antibodies specific to the immunized antigen, HbF.

For the rest of the immunized mice, the IgG and IgM expressing cells were isolated from spleen cells prior to the cell fusion. By this cell fractionation, the spleen cells were separated into three populations, i.e., IgM expressing cells (IgM^+ cells), IgG expressing cells (IgG^+ cells) and IgM and IgG negative cells (IgM^-/IgG^- cells). All cell populations were separately fused with myeloma cells. After fusion, cells at the same cell concentration were spread into 96 well plates. Generated hybridomas were determined and culture supernatants from hybridoma containing wells were screened for antibody isotypes (IgM or IgG) and anti-HbF activity by indirect ELISA. The results are shown in Tables 2 and 3.

In three independent experiments, using each cell fraction in cell fusion, resulted in percentage yields of 32 ± 9%, 35 ± 19% and 29 ± 14% (mean ± SD) of seeded wells contained hybridomas obtained from IgM^+, IgG^+ and IgM^-/IgG^- cell populations (Table 2). These results indicated that the isolated IgM and IgG expressing cells could be used as fusion partners and that there was no difference in fusion efficiency.

All hybridoma culture supernatants obtained were screened for their antibody isotypes. By using IgG^+ cells, in three independent experiments, the percentages of hybridomas producing IgG isotype antibody ranged from 86 to 11% (41 ± 40%; mean ± SD) (Table 3). Interestingly, none of the tested hybridomas produced IgM antibody. A large number of hybridomas, however, were non-IgG or IgM secreting cells (Table 3). By this fusion, more than 50 hybridoma clones producing HbF specific IgG mAbs were obtained. In contrast, when IgM^+ cells were used for cell fusion, the majority of the obtained hybridomas produced the IgM isotype antibody (85 ± 7%) (Table 3). Only 4 ± 3% of the obtained hybridomas produced the IgG antibody and 2 ± 3% were classified as non-IgG or IgM producing cells (Table 3). For the small number of the obtained hybridomas producing IgG isotypes it was presumed that during magnetic cell sorting for IgM^+ cells, a small amount of IgG^+ cells was contaminated in the IgM^+ sorted cells. When such IgG^+ cells are placed into any wells, the wells will contain IgG producing hybridoma. Therefore, we could expect to obtain some wells containing IgG isotype. 9 ± 3% of tested hybridomas produced antibodies that were determined as IgG and IgM isotypes (Table 3). We assumed that, in these wells, there was more than one hybridoma clone which produced IgG and IgM in the same wells. From the IgM^+ cell fusion, a total of more than 50 hybridoma clones producing HbF specific mAbs and having the IgM isotype were obtained. When the negative cells (IgM^-/IgG^- cells) were used, more than 90% of the generated hybridoma clones were non-IgM or IgG producing cells (Table 3). Our results indicated that spleen cells expressing IgM and IgG antibody can be used to generate hybridomas producing IgM or IgG antibodies, respectively.

Table 2 Hybridomas generated from IgM^+ cells, IgG^+ cells and IgM^-/IgG^- cells

Cell fractions	Seeded wells	Number of hybridoma containing wells	% Of hybridoma containing wells
IgM^+ cells			
Exp. 1	960	418	44
Exp. 2	576	177	31
Exp. 3	768	161	21
IgG^+ cells			
Exp. 1	288	155	54
Exp. 2	136	57	42
Exp. 3	328	28	9
IgG^-/IgM^- cells			
Exp. 1	384	48	13
Exp. 2	960	447	47
Exp. 3	960	255	27

Spleen cells from immunized mice were fractionated into IgM^+ cells, IgG^+ cells and IgM^-/IgG^- cells. Each cell fraction was fused with myeloma cells, adjusted to the same concentration and seeded into 96 well plates. The generated hybridomas were observed with an inverted microscope. Three independent experiments (Exp. 1, Exp. 2 and Exp. 3) were carried out

Resting B cells express two classes of membrane bound immunoglobulins, IgM and IgD, that function as the receptors for antigens. Binding of specific antigens to the antigen receptors, in the presence of appropriate cytokines from helper T cells, induces B cell proliferation and differentiation into antibody producing cells (Lanzavecchia and Sallusto 2009; Abbas and Lichtman 2007). During B cell

Table 3 Antibody isotypes produced by the hybridomas generated from IgG^+ cells, IgM^+ cells and IgM^-/IgG^- cells

Cell fraction	Antibody isotype	Experiment 1		Experiment 2		Experiment 3	
		Number of positive wells/total hybridoma containing wells	% Of total hybridoma containing wells	Number of positive wells/total hybridoma containing wells	% Of total hybridoma containing wells	Number of positive wells/total hybridoma containing wells	% Of total hybridoma containing wells
IgG^+ cells	IgG	133/154	86	0/57	25	0/28	11
	IgM	0/154	0	14/57	0	3/28	0
	IgG and IgM	0/154	0	0/57	0	0/28	0
	None	21/154	14	43/57	75	25/28	89
IgM^+ cells	IgG	35/418	8	173/177	0	146/161	3
	IgM	312/418	75	0/177	90	4/161	91
	IgG and IgM	46/418	11	3/177	10	11	7
	None	25/418	6	1/177	0.6	161	0
IgM^-/IgG^- cells	IgG	5/48	10	0/477	0	0/255	0
	IgM	0/48	0	0/477	0	0/255	0
	IgG and IgM	0/48	0	0/477	0	0/255	0
	None	43/48	90	477/477	100	255/255	100

Spleen cells from immunized mice were fractionated into IgG^+ cells, IgM^+ cells and IgM^-/IgG^- cells. Each cell fraction was fused with myeloma cells, adjusted to the same concentration and seeded into 96 well plates. Culture supernatants were taken from the hybridoma containing wells of each cell fraction and determined for antibody isotypes by ELISA. Results of the three independent experiments are shown

differentiation, heavy chain class switching in some activated B cells occurs and results in a change from production of IgM isotype to other isotypes. As the spleen is the major site for the production of antibodies, after appropriate antigen stimulation, the spleen contains B cells carrying various types of membrane immunoglobulins. In the present report, we isolated B cells carrying IgM or IgG and employed these to generate hybridoma cells by the conventional hybridoma technique. We found that the obtained hybridomas produced corresponding antibody isotypes as were expressed on the surface of their starting cells. This finding, therefore, can be employed to conquer the drawback of standard hybridoma procedures that produce unexpected isotype of mAbs.

In conclusion, in the present report, we introduce a technique for producing of mAbs having a desired isotype. The developed technique is potentially valuable for purposes that require a specific isotype of monoclonal antibody.

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