

A Unique Report: Development of Super Anti-Human IgG Monoclonal with Optical Density Over Than 3

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ABSTRACT

Purpose: Monoclonal antibodies and related conjugates are key reagents used in biomedical researches as well as, in treatment, purification and diagnosis of infectious and non-infectious diseases. **Methods:** Balb/c mice were immunized with purified human IgG. Spleen cells of the most immune mouse were fused with SP2/0 in the presence of Poly Ethylene Glycol (PEG). Supernatant of hybridoma cells was screened for detection of antibody by ELISA. Then, the sample was assessed for cross-reactivity with IgM & IgA by ELISA and confirmed by immunoblotting. The subclasses of the selected mAbs were determined. The best clone was injected intraperitoneally to some pristane-injected mice. Anti-IgG mAb was purified from the animals' ascitic fluid by Ion exchange chromatography and then, mAb was conjugated with HRP. **Results:** In the present study, over than 50 clones were obtained that 1 clone had optical density over than 3. We named this clone as supermonoclonal which was selected for limiting dilution. The result of the immunoblotting, showed sharp band in IgG position and did not show any band in IgM&IgA position. **Conclusion:** Based on the findings of this study, the conjugated monoclonal antibody could have application in diagnosis of infectious diseases like Toxoplasmosis, Rubella and IgG class of other infectious and non-infectious diseases.

Introduction

Monoclonal antibodies can be produced in specialized cells through a method now popularly known as hybridoma technology.¹ Hybridoma technology was first invented by two scientists, Georges Kohler and Cesar Milstein. From 1975, Köhler and Milstein successfully fused antibody-producing mouse spleen cells with mouse myeloma cells, the fusion of somatic cells has been carried out for many years with a variety of different aims and this technique quickly became one of immunology's key technologies.² Using hybridoma technology, monoclonal antibodies have been prepared against a wide range of antigens including growth factors, growth factor receptors, viruses, bacterial products, hormones and differentiated antigens.³

In fact, monoclonal antibodies (mAbs) have been widely applied in various fields such as diagnosis applications, purification, disease monitoring, identifying prognostic markers and therapy.⁴ For most research, diagnostic and therapeutic purposes, monoclonal antibodies derived from a single clone and thus specific for a single epitope, are preferable.⁵ In most diagnostic kits of the infectious diseases, for instance, Rubella, H.pylor Toxoplasmosis and etc, the

conjugated monoclonal antibodies against human IgG carry out as a key role. Due to in this need, generation of mAb seems invaluable. To approach these goals, generation and characterization of a highly specific mAb against human IgG was investigated.

The prim aim of this study including: production and application of mabs against human IgG for development of diagnostic kits and large scale, semi-industrial production and standardization of this product towards self-sufficiency of the country.

Materials and Methods

Immunization Procedure and Screening of Immunized Animals

Four female Balb/c (6-8 weeks old) mice were used for purified human IgG (Affinity Purified Human IgG, Sigma) immunization. Each mouse was immunized 4 times with an interval of two-three weeks subcutaneously. The first Immunization was performed using Freund's complete adjuvant (Sigma-Aldrich Co. Louis, USA). Incomplete Freund's adjuvant (Sigma-Aldrich Co. Louis, USA) was used for the 2nd, 3rd and 4th immunization. For the first immunization, 50 µg of purified human IgG was mixed with an equal volume

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of Freund's complete adjuvant. For the subsequent immunizations 50 µg purified human IgG were injected with Freund's incomplete adjuvant. Three days before the cell fusion, 50 µg of antigen (without any adjuvant) was injected intravenously.

A week after the second injection, blood was taken from each mouse by a vertical incision of the tail vein and the antibody response was measured by ELISA. The mouse with the highest serum antibody titre was selected as the spleen donor. Sera collected from non-immunized and immunized mice served as negative and positive controls.

Cell Fusion and Hybridoma Production

Three days after final immunization, spleen of the immunized mouse was aseptically removed and fused with SP2/0 myeloma cell line at a ratio of 1:5 (1 SP2/0 and 5 spleen cells) by PEG (polyethyleneglycol, MW 1450, Sigma) as fusogen. Selective HAT medium (Gibco) was added to the fused cells and cells were seeded into five 96-well microtitre plates (Nunc) containing feeder layer. The cells were incubated at 37 °C with 5% CO₂ for 2-3 days. Cell growth and colony formations were examined daily. Colonies were appeared after 5-7 days. Once the colony diameter reached to 1 mm the presence of antibody against the immunized antigen was determined by ELISA method. Then, positive hybridomas were selected for further studies.

Cloning of Hybridoma Cells by Limiting Dilution Assay

After screening, the clones with high absorbance were selected for cloning by Limiting Dilution (L.D) method. The cells were diluted so that contained only one cell in each 10 µl. Twelve days after L.D, the supernatants of monoclones were screened for production of antibody. Suitable monoclones possessing high absorbance were selected for further characterization and considered for mass production.

Assessment of Anti-IgG Cross-Reactivity with other Classes of Immunoglobulins by a Sandwich ELISA

In order to determine the cross reactivity with IgM & IgA, the micro titer plates were coated with each class of the purified immunoglobulins (5µg/ml in coating buffer, 100µl/well) and incubated for 1.5 hour at 37 °C. Plates were saturated with 2% BSA at 37 °C for 1.5 hr. Wells were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) for 5 min. At the next step, Hybridoma culture supernatants with distinct dilution was added to the wells and incubated as described above. Then, 100 µl of 1:4000 dilution of HRP-conjugated rabbit anti-mouse IgG (Sigma-Aldrich Co. Louis, USA) was added to the wells and incubation was continued for 1.5 hrs at 37 °C. After washing, 100 µl of Tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated at room temperature in a dark place. After 15 min, the reaction

was stopped by adding 100 µl of stopping solution (0.16 M H₂SO₄) to each well. The Optical Density (OD) of the reactions was measured at 450 nm by an ELISA reader (STAT FAX 303+). In the end of the reaction and by reading their absorbance, cross reactivity of the monoclonal antibodies with IgM & IgA was determined.

Isotype Determination

The class and subclass of the mAbs were determined by an ELISA with a mouse monoclonal sub isotyping kit containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, following the procedure provided by the manufacturer (Thermo, USA).

Production of Ascitic Fluids

Hybridoma cells producing IgG mAbs were grown in RPMI₁₆₄₀ supplemented with 10% fetal bovine serum, harvested and washed twice in PBS (pH 7.2). Ten days after pristane injection, 6-8 weeks old Balb/c mice were injected intraperitoneally with 2-3×10⁶ hybridoma cells suspended in 0.5 ml PBS (pH 7.2). Fluid was collected from the peritoneal cavity 8 to 10 days after the injection of the cells, by using of needle 19. Ascitic fluid was kept at 4 °C for 1 h and centrifuged at 5000 g for 15 min. Supernatant was collected and stored at -20 °C until used.

Antibody Purification

The monoclonal antibodies were purified from ascitic fluid by Ion exchange chromatography based on its isotype. Briefly, ascitic fluids were filtered through 45 µm filter and were diluted 1 to 2 with PBS and fractionated with 40% saturated ammonium sulfate. After several times of washing with 40% ammonium sulfate, the fraction was centrifuged for 15 minutes in 5000g. The precipitated fraction was dialyzed against 0.05 M phosphate buffer pH 7.4 containing 0.05 M NaCl. The final dialyze was exchanged against the column washing buffer (Tris 40 mM). Purification of ascitic fluid was done by Ion exchange chromatography (DEAE-Sepharose 6B) which is a simple and economical method. At first, the column was eluted with washing buffer (Tris 40 mM, pH 8.1) in order that the pH of the external buffer to be the same as the pH of internal buffer. Then the dialyzed sample in 60 mg/3ml concentration was run to the column with dimensions of 1.6×15cm. Distinct antibody was eluted from the column through washing buffer containing 50 mM NaCl, and the fractions were collected in 5 ml/20 ml. Finally, the purified fractions were kept for conjugation with Peroxidase.

SDS-PAGE Gel Electrophoresis

Confirmation of the mAb purity was monitored by SDS-PAGE in reduced and non-reducing condition. In addition, to check the result of cross reactivity with IgM & IgA by ELISA method this technique was used. The samples (purified mAb, purified IgG, IgM&IgA)

were mixed with sample buffer, then boiled for 2 - 5 min and cooled on ice. Electrophoresis was done in a 12.5% SDS-PAGE gel with a mini- PROTEAN electrophoresis instrument (Bio- Rad Laboratories, Hercules, CA, USA) 100 mA for 1 hr. The gel was stained with Coomassie Brilliant Blue R-250 (Sigma).

Western Blotting

Western blotting technique was used for confirming the result of ELISA and to see pattern of specificity and cross -reactivity of anti-IgG monoclonal antibody.

Briefly, the nitrocellulose membrane and several thicknesses of Whatman chromatography papers were soaked in the transfer buffer (25 mM Tris, 192 mM glycine, 20% V/V methanol, pH 8.3). The wet nitrocellulose membrane was overlaid on the wet Whatman sheets by taking precaution to avoid bubbles. Then, the gel of SDS-PAGE was placed on the wet nitrocellulose membrane and then several wet Whatman papers were placed on it. Transfer of the proteins from gel to nitrocellulose membrane was done in 100V for 3 hours. Then, non- specific sites were blocked with 2% BSA solution. After three times of washing, the membrane was cut into strips and incubated for 2 hours at 37 °C with the supernatants of suitable clones. Again, after five times of washing, the strips were incubated for 2 hours at 37 °C with Rabbit Anti- Mouse IgG conjugate (1/2000 dilution). The strips were washed and detected by ECL (Amersham Phamacia Biotech Inc, USA) hyperfilm after exposure for 5 min.

Labelling of Monoclonal Antibody with Horseradish Peroxidase Enzyme (HRP)

For conjugation, the Nakan and Periodate methods were used. Briefly, 4 mg of HRP was dissolved in 1 ml of distilled water. Then 0.2 ml freshly prepared sodium Periodate solution (0.1M) was added to the enzymatic solution and incubated on shaker for, 20 minutes at room temperature. The solution was dialyzed against acetate buffer (pH 4.4), overnight at 4 °c. 8 mg of the purified monoclonal antibody was dissolved in 1 ml sodium carbonate (10 mM, pH 9.5).The pH of the dialyzed enzyme was reached to 9 and immediately the solution containing monoclonal antibody was added to it and shaken for 2 hours at room temperature. Then 0.1 ml of the freshly prepared sodium brohydrate was added and incubated for 30 minutes at room temperature. The final solution was precipitated with ammonium sulfate and then was dialyzed against PBS buffer.

Results

Among the five immunized Balb/c mice against human IgG, the serum of the immune mouse at 1/32000 dilution, indicated the highest absorbance in reaction with human IgG using ELISA method. So the immune mouse was selected for the fusion. The final result of the successful fusion of the immune mouse spleen cells

with myeloma SP2/0 cells were about 50 wells containing positive clones with high absorbance in reaction with IgG that 1 clone had optical density over than 3. We named this clone as supermonoclonal (Figure 1) and was selected for cloning by limiting dilution (L.D) method. The yield of limiting dilution was many clones with absorbance over than 3 and about 2 at 0.01 dilutions which did not show any cross-reactivity with IgM and IgA by ELISA method (Table 1).

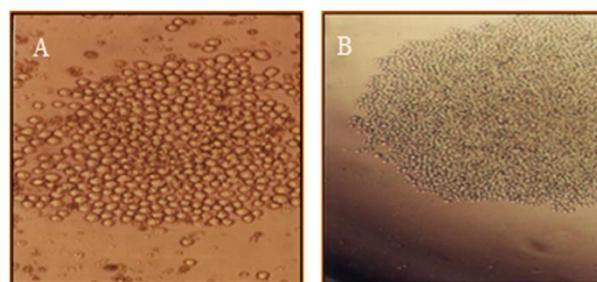


Figure 1. Proliferated suitable mono clone (supermonoclonal) selected for injection into the peritoneum of mice. Supermonoclonal in the growing form (Mag.10X) (A), Supermonoclonal in the highly proliferated form (Mag.4X) (B).

Table 1. Comparison of the mean absorbance of supernatant of the supermonoclonal at 450 nm.

	IgG	IgM	IgA
SP2/0 (N.C)	0.05	0.07	0.06
Mouse Serum (N.C)*	0.1	0.08	0.09
Immune mouse serum (P.C)**	1.1	0.2	0.2
Super monoclonal	>3	0.21	0.18
Supermonoclonal(1/100 dilution)	2	0.11	0.1
*negative control with 1/32000 dilution, **positive control with 1/32000 dilution			

Isotype of this mAb was identified as IgG1 and k light chain (Table 2). For large scale production of monoclonal antibody, super monoclonal related hybridoma cells were injected into the peritoneum of the Balb/c mice, which have previously been primed with 0.5ml pristane. After 7-10 days, approximately 5 ml ascitic fluid was harvested from the peritoneum of each mouse. Ascitic fluid purified with Ion exchange chromatography, and conjugated with HRP. There was 30 mg protein in ascitic fluid that reduced to 7.5 mg antibody after purification step. The result of purification confirmed with SDS-PAGE and PAGE. In SDS PAGE, two bands of 50 kDa and 25 kDa were appeared that demonstrator heavy and light chains. In PAGE, only one 150 kDa band was appeared that demonstrator of purified antibody (Figure 2). The specificity and cross reactivity of the anti-IgG was also confirmed by an Immunoblotting procedure (data not show). The titer of mAb in ascitic fluid was examined by ELISA method and the results showed that its

1/100,000 dilution has high absorbance with IgG (above 1) but has no absorbance with IgM & IgA (Table 3). Also, the results of conjugation indicated that

1/64000 dilution of conjugate had high absorbance with IgG (above 1) and didn't show any cross reactivity with IgM & IgA (Table 3).

Table 2. Results of subclass isotyping of mouse mAb at 450 nm.

	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM	Kappa	Lambda
Mouse anti-human IgG	1.101	0.131	0.154	0.168	0.117	0.136	1.692	0.138

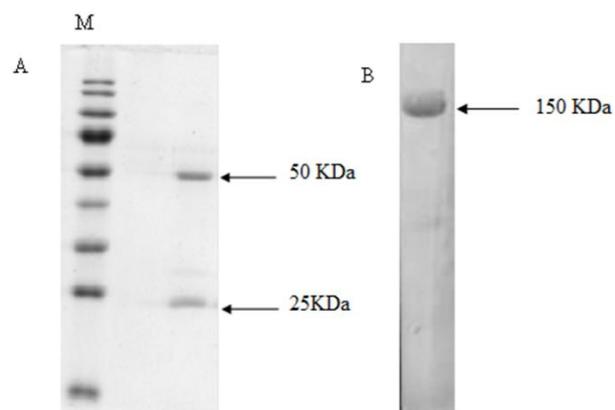


Figure 2. SDS- PAGE of purified monoclonal antibody. In reduced form (A), two bands were seen in 50 & 25 kDa but in non-reduced condition (B), only one band was seen in about 150 kDa.

Table 3. Comparison of the mean absorbance and cross reactivity of ascitic fluid and anti- human IgG conjugate at 450 nm.

	IgG	IgM	IgA
SP2/0 (N.C)	0.05	0.07	0.06
Mouse Serum (N.C)*	0.1	0.08	0.09
Immune mouse serum (P.C)**	1.1	0.18	0.12
Ascitic fluid (1/100000 dilution)	1.2	0.09	0.08
Mouse anti- human IgG conjugate (1/64000 dilution)	1.4	0.07	0.06
*negative control with 1/32000 dilution, **positive control with 1/32000 dilution			

Discussion

Hybridoma technology has been rapidly and successfully applied to a wide variety of biological problems of both theoretical and practical importance.⁶ MAbs are therefore of enormous utility in applications such as experimental biology, medicine, biomedical research, diagnostic testing, and therapy.⁷ More importantly, each antibody is highly specific for a particular antigen; this characteristic feature of antibodies has led to their routine usage in diagnostic kits.⁸

IgG antibodies are involved in predominantly the secondary immune response.⁹ The presence of specific IgG, in general, corresponds to maturation of the antibody response. In addition, in chronic condition of infectious diseases; the class of the produced antibody against pathogen is IgG.¹⁰ Therefore monoclonal anti-human IgG is very important, significant and as a key reagent for its recognition. Accordingly, production of monoclonal antibody without any cross reactivity with homologous molecules such as other classes of immunoglobulins can be used in diagnostic kits of infectious diseases.¹¹

When mAbs are produced, it is important to consider antigen features, which include the quality and quantity of the antigen and the antigen preparation. The specificity of the immune response obtained depends on the purity of the antigen applied.¹² Therefore in this study, purified human IgG immunoglobulins were used to immunize Balb/c mice. Upon such procedure, we observed high immunologic response in the immunized mouse, whose serum resulted in absorbance above 1 with 1/32000 dilution and the most immune mouse was selected for the fusion.

In similar previous study, Majidi et al produced monoclonal antibody against human IgE which could be used in designing ELISA kits. Purified IgE was used for immunization, in order to produce mAb.¹³

In a study, YÜCEL et al produced hybridomas against Hepatitis B virus (HBV) by fusing spleen cells from hyper immunized mice with SP2/O mouse myeloma cell. HBsAg proteins were used for immunization then; they concluded that these mAbs were valuable for diagnosis of hepatitis B surface antigen in human serum.¹ In all of these studies, native proteins purified from the natural source were preferred for its most likely to produce useful antibodies and high concentrations of mAbs produced.

In the present study, the best clone designated as supermonoclonal, was injected intraperitoneally to pristane-injected mice. The production of monoclonal antibody in the ascitic fluid is commercially cost effective also; a rapid, reproducible method for large-scale production in comparison with expensive and time-consuming culture methods.¹⁴ Anti-IgG mAb was purified from the animals' ascitic fluid by Ion exchange chromatography. The purity of the sample was tested by SDS-PAGE. Immunoblotting and ELISA were done and the results showed that the harvested antibody

recognizes human IgG and there was no cross-reactivity with other classes of immunoglobulins. Furthermore, anti-IgG mAb was interacted with human IgG with a very high specificity and affinity. These mAbs with high affinity are useful tools for quantitation of human IgG subclass levels in various diseases. So, this mAb could be a useful tool for use in the development of diagnostic kits based on sandwich ELISA. Taking all together, the conjugated monoclonal antibody could have application in diagnosis of infectious diseases like Toxoplasmosis, Rubella, H.Pylori and IgG class of other infectious and non-infectious diseases. Importantly, production of purified monoclonal antibody and HRP conjugated IgG could be consider another step toward Iran self-sufficiency.

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Conflict of Interest

The authors report no conflicts of interest.

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