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Mass-Production and Characterization of Anti-CD20 Monoclonal Antibody in Peritoneum of Balb/c Mice

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ABSTRACT

Purpose: Monoclonal antibodies are important tools are used in basic research as well as, in diagnosis, imaging and treatment of immunodeficiency diseases, infections and cancers. The purpose of this study was to produce large scale of monoclonal antibody against CD20 in order to diagnostic application in leukemia and lymphomas disorders. ***Methods:*** Hybridoma cells that produce monoclonal antibody against human CD20 were administered into the peritoneum of the Balb/c mice which have previously been primed with 0.5 ml Pristane. After twelve days, approximately 7 ml ascetic fluid was harvested from the peritoneum of each mouse. Evaluation of mAb titration was assessed by ELISA method. In the present study, we describe a protocol for large scale production of MAbs. ***Results:*** We prepared monoclonal antibodies (mAbs) with high specificity and sensitivity against human CD20 by hybridoma method and characterized them by ELISA. The subclass of antibody was IgG2a and its light chain was kappa. Ascetic fluid was purified by Protein-A Sepharose affinity chromatography and the purified monoclonal antibody was conjugated with FITC and Immunofluorescence was done for confirming the specific binding. ***Conclusion:*** The conjugated monoclonal antibody could have application in diagnosis B-cell lymphomas, hairy cell leukemia, B-cell chronic lymphocytic leukemia, and melanoma cancer stem cells.

Introduction

The current era of monoclonal antibodies (MAb) was introduced with the hybridoma technology devised by Köhler and Milstein in 1975.[1] They fused a single antibody-producing cell from the spleen of an immunized mouse with a human myeloma cell to produce an immortal hybrid cell that secreted an antibody to a single antigenic epitope. The antibody-producing spleen cell provided the specificity and the myeloma cell provided the immortality.[2] The first monoclonal antibodies were primarily mouse monoclonal and used to identify antigens on cells, usually by immune-fluorescent techniques, particularly by automated flowcytometry. Currently, monoclonal antibodies play crucial roles in diagnosis applications, disease monitoring, identifying prognostic markers and therapy. Meanwhile each antibody is highly specific for a particular antigen; this characteristic feature of antibodies has led to their routine usage in diagnostic kits and in uncovering the function of such antigens in a number of physiological and pathological conditions.[3,4] By hybridoma technology, monoclonal antibodies have been prepared against an extensive range of antigens including growth factors, growth factor receptors, mutated antigens, viruses, bacterial products, hormones, drugs, enzymes, and differentiated antigens. Such antibodies are used commonly in the identification of the antigens in human tumor biopsies and sera, and in considering their role in tumor progression.[5]

CD20, a 33–36 KD non-glycosylated phosphoprotein that has stable expression and tightly bound to the membrane with little modulation during maturation, and it likely has an important role in B-cell activation and regulation of cell cycle. All these features make CD20 an ideal anti-B-cell target.[6-8] CD20 is expressed within key B-cell development stages that give rise to B-cell lymph-proliferative disorders. production of the first anti-CD20 monoclonal antibody could have application in diagnosis malignancies, disease monitoring, prognostication or therapy of B-cell lymphomas, hairy cell leukemia, B-cell chronic lymphocytic leukemia, and melanoma cancer stem cells.[9,10]

For mass-production of the monoclonal antibody, hybridoma cells should be reproduced in either of two methods: in vivo method; Injection of desired clone

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into the peritoneal cavity of the mouse or in vitro method; Culture of the cells in tissue culture flasks. In this regards, the required purity and concentration of mAb were obtained in mouse ascetic fluid and tissue-culture supernatant.[11]

When hybridoma cells are injected into the peritoneum of mouse, the cells grow and produce ascitic fluid.[12] The method of ascitic fluid production in peritoneum of mouse is economic, but reproduction of cells by in vitro method needs special skills, special medium.[13] Furthermore, the loss of antibody's original glycosylation by in vitro culture method affected mAb biologic functions like increased immunogenicity, reduced binding affinity, accelerated clearance in vivo and preferred pharmacokinetic characteristics. Furthermore, contaminated hybridoma cells producing mAb with infectious disease often be passed through mice. So, antibody production in ascitic fluid can be a suitable and economical method.[14] The diagnostic-industry scale of mAb production is usually small to medium and large. The therapeutic industry is significantly less concerned than the diagnostic commerce with cost and turn-around time, and its production scale is medium to large. In summary, by the in vivo method, hybridoma cells in high density allow abundant production of highly active and pure MAbs necessary for certain specific purposes.

Materials and Methods

Female BALB/c mice (4-6 weeks old) were obtained from Pasteur institute of Iran. 0.5 ml Pristane (2, 6, 10, 14 tetra methyl pentadecane, Sigma) was administered intra-peritoneally into each mouse. Twelve days after priming with Pristane, high cell densities of a desired mono clone (1×10^6 cells / 0.5 ml PBS) were injected intra-peritoneally into each mouse. The mice were assessed daily for production of ascetic fluid after the injection of hybridoma cells. Abdomen of the mice was completely enlarged and their skins were extended about ten days after the injection of cells. Their ascetic fluids were harvested by 19-gauge needle. After 4 days, ascitic fluid of the mice were harvested again and centrifuged and the related supernatants were collected for characterization.[15]

The titer of monoclonal antibody was assessed by ELISA method. In this assay, 100 µl BSA-conjugated peptide (10 µg/ml) was coated in 96 wells plate (Nunc) overnight at 4 °C. After twice washing with PBS-Tween 20 (0.05%), non-specific sites were blocked with 2% BSA and incubated at 37 ºC for 45 minutes. The washing was repeated and then 100µl of the continuous dilution of ascitic fluid was added to each well and incubated for an hour at 37 °C. Then the plate was washed five times. 100 µl of Rabbit Anti-mouse Ig conjugated with HRP (100 µl, 1/4000 dilution) (Sigma-Aldrich Co. Louis, USA) was added to each well and incubated for 45 minutes at 37 °C. After five times of washing, 100 µ1 of Tetramethylbenzidine (TMB) substrate solution (Sigma) was added into each well and incubated for 20 minutes in dark place at RT. After 20 min, the reaction was stopped by adding 100 µl stopping solution (0.16 M H2SO4) to each well and Optical Density (OD) was read by ELISA Reader at 450 nm. Therefore the titer of monoclonal antibody in ascitic fluid was determined.[16]

Isotyping

ELISA mouse mAb isotyping Kit (Thermo, USA) was used for determination the class and subclass of the mAbs. First, tris buffer saline (TBS) was used for 1/50000 dilution of the ascetic fluid and 50 µl of diluted antibody added to each well of the 8-well strip. At the next step 50 µl of the anti-mouse IgG + IgA+ IgM + HRP conjugated was added to each well of the 8-well strip and then the plate was incubated for an hour at room temperature. After 3 times washing, 75 µl of TMB substrate was added to each well and the plate was incubated at room temperature in a dark place. After 10 min, the reaction was stopped by adding 75 µl of stopping solution to each well. The subsequent color of the reactions was measured at 450 nm by an ELISA reader (Biotech, USA).[17]

Purification

The diluted ascetic fluid in PBS (1:2) was precipitated with saturated ammonium sulfate and dialyzed against PBS pH 7.4 and IgG2a class mAbs were purified by Protein-A-Sepharose column affinity chromatography. Briefly, ascetic fluids were filtered through 45 µm filter and pH was adjusted to pH 7.5. Antibodies were affinity purified using a column of Protein A Sepharose. The elution was performed using 0.1 mol/l glycine buffer, pH=2.7. Mouse IgG 2a elute with 0.1M citrate buffer in pH 4.5. The eluted antibodies were dialyzed overnight against PBS pH=7.5 and the reactivity of the antibodies were measured by ELISA as described above.

Confirmation of the MAb purity by SDS-PAGE

Confirmation of the MAb purity was monitored by SDS-PAGE in non-reducing condition. 10 µg of purified mAb was mixed with 10 µl of sample buffer, then boiled for 2-5 min and cooled on ice. Electrophoresis was done in a 12% 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).

Conjugation of Monoclonal Antibody with Fluorescein Isothiocyanate (Fitc)

For conjugation, 200 µl mAb (5mg/ml) was added in 800 µl Reaction Buffer (500 Mm Carbonate, pH = 9.2) and dialyzed against PBS buffer in 24 hours. The antibody concentration was measured after buffer equilibration in 280 nm. 10 mg of FITC was dissolved in 1 mL anhydrous DMSO immediately before use. FITC (SIGMA, Germany) was added to give a ratio of

80 µg per mg of antibody and mixed immediately. The tubes were wrapping in foil then incubate and rotate at room temperature for 1 hour. The unreacted FITC was removed and exchanged the antibody into Storage Buffer (10 mM Tris, 150 mM Nacl, 0.1% Nah3, pH=8.2) by dialysis during overnight.[5]

Direct Immunofluorescence Staining

This technique was used for confirming the result of conjugation method. Raji cell line as a positive control (CD20 +) and Molt-4 as a negative control (CD20 -) were cultured in microtiter plates and after reaching in 50% confluence, cell suspension were transferred to 15 mL conical tubes and washed with RPMI medium. 50 µL of the cell suspension (1×10^6 cells) was Added to each microtiter plate well and then 1/1000 dilution of fluorochrome-conjugated monoclonal antibody was added. The mixture was incubated for 45 minutes on ice and washed two times with 100 µL of cold PBS buffer. Cells were suspend in 200 µL of 3.7% formaldehyde solution for fixation of Raji cells in 10 min at Room temperature and after two times washing, stained cells were analyses by florescent microscope.[5]

Results

For priming the peritoneum of the mice, Pristane were administered. High cell densities of a desired mono clone (1×10^6 cells) were suspended in 0.5 ml of sterile PBS and injected to each mouse. About 5 ml ascetic fluid was harvested from each mouse after twelve day. About 2 ml ascetic fluid was harvested from their peritoneum for a second time, after 4 days. The titer of monoclonal antibody in ascetic fluid was assessed by ELISA method. The mean absorbance of non-immune mouse serum, Immune mouse serum, and ascetic fluid was compared in Table 1 at 450 nm.

Table 1. Comparison of the mean absorbance of ascetic fluid at 450 nm

NC (SP/0)	NC* (Non Immune mouse serum)	PC* (Immune mouse serum)	Ascetic fluid (1/16000 dilution)
0.07	0.1	1.2	1.08
* With 1/8000 dilution			

The results showed that its 1/16000 dilution has high absorbance with CD20 antigen (above 1). Determination of mAb class and subclasses in ascetic fluid was examined by mouse isotyping kit (Thermo, USA). The subclass of monoclonal antibody was IgG2a with "kappa" type light chain. The product was precipitated by saturated ammonium sulfate and dialyzed against PBS and assess by UV at 280 nm. 40 mg concentrated protein was harvested. Purification by Protein-A-Sepharose column affinity chromatography yielded about 5 mg of monoclonal antibody and only one 150 KD band was appeared in non-reducing SDS-PAGE Figure 1. The purified monoclonal antibody was conjugated with fluorescein isothiocyanate (FITC) and then Direct Immunofluorescence Staining was used for confirming the result of conjugation method and mAb specification. Specific attachment of purified mAb with CD20 antigen was evaluated by immuno-floresance techniques in the completely.

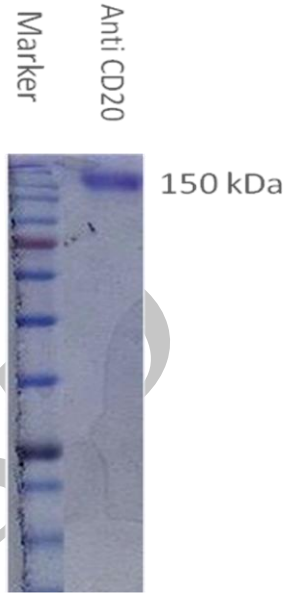

Figure 1. Non-reducing SDS-PAGE, one 150 KD band was appeared which could confirm purified antibody product.

Discussion

All branches of medical sciences have been touched with the hybridoma technology, in diagnosis malignancies, disease monitoring, prognostication or therapy.[18] Currently, monoclonal antibodies play crucial roles in diagnosis applications, disease monitoring, identifying prognostic markers and therapy. Since the introduction of the hybridoma technology by Kohler and Milstein in 1975, a variety of methodological technologies have been developed for large-scale production of mAbs.[1] To produce the desired mAb, the cells must be grown in either of two ways: by injection into the abdominal cavity of a suitably prepared mouse or by tissue culturing cells in plastic flasks. For efficient laboratory-scale production of monoclonal antibodies, hybridoma cells are injected into the peritoneum of mouse; the cells grow and produce ascetic fluid.[2] The production of monoclonal antibody in the ascetic fluid is commercially cost effective for large-scale production in comparison of expensive and time-consuming culture methods. In mouse method, two important factors for producing the required amount of cells are injected Pristane and the interval of priming with hybridoma cells. For example, for harvesting about 3-4 ml ascetic fluid from the peritoneum of each mouse, one million cells must be injected to the peritoneum of mouse.

In similar previous study, Baradaran et al produced large scale monoclonal antibody against EGFR in ascetic fluid efficiently and 10.4 mg antibody was purified with Ion exchange chromatography (IEC).[16]

In other study Galen et al used in vivo method for mass production of monoclonal antibodies against human rennin that ascites were produced after intra-peritoneal injection of cloned hybridoma cells into pristane-treated Balb/c mice. Then monoclonal antibodies were purified from ascites by affinity chromatography on protein A Sepharose.[19] Furthermore Mittal et al. used the same in production and characterization of murine monoclonal antibodies against *Haemophilus parasuis*.[20] In all these studies, In vivo methods were preferred for its cost effectiveness and high concentrations of mAbs produced.

Since analysis of mAb produced in tissue culture reveals that a desired antibody function is diminished or lost. In vitro methods are expensive and time-consuming and often fail to produce the required amount of antibody even with skilled manipulation.[8,9] Considering to all issues, in this study injection into the abdominal cavity was preferred. Pristane were used for priming the peritoneum of the mice by high cell densities of a desired mono clone (1×10^{6} cells). Ascetic fluids were harvested from the mice and the titer of monoclonal antibody was assessed by ELISA method. The results showed that 1/16000 dilution has high absorbance with CD20 antigen (above 1).

For the class and subclass of mAbs determination, ELISA mouse mAb isotyping Kit was used and Monoclonal antibody class was IgG2a and its light chain was "kappa" type. Concentration of the dialyzed ascetic fluid product in assay with UV at 280 nm was about 40 mg and Protein-A Sepharose purification with affinity chromatography yielded about 5 mg of purified monoclonal antibody. Purity was monitored by SDS-PAGE in non-reducing condition, only one 150 KD band was appeared that demonstrate purified antibody. The purified monoclonal antibody was conjugated with fluorescein isothiocyanate (FITC). Direct Immunofluorescence Staining was used for confirming the result of conjugation method and mAb specification. Specific attachment of purified mAb with CD20 antigen was evaluated using immuno-floresance techniques in the surface of Raji cells (shown in Figure 2). CD20 is expressed within key B-cell development stages and generation of the first anti-CD20 monoclonal antibody could have application in diagnosis malignancies, disease monitoring, prognostication or therapy of B-cell lymphomas, hairy cell leukemia, B-cell chronic lymphocytic leukemia, and melanoma cancer stem cells.[18]

Conclusion

Taking all together, ascetic fluid production method seems to be a reasonable and economic approach for mAb production with suitable purity and concentration

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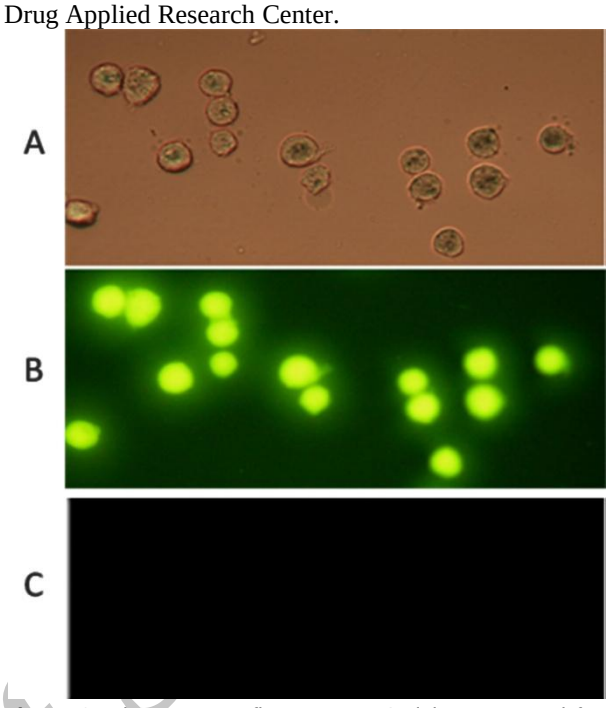

Figure 2. Direct Immunofluorescence Staining was used for confirming specific attachment of purified mAb with CD20 antigen in the surface of Raji cells. A. Raji cell before treatment, B. after treatment with purified mAb, C. Negative control.

Conflict of Interest

There is no conflict of interest in this study.

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