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Production and Purification of Monoclonal Antibody Against Tumor Marker of TPA

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ABSTRACT

Considering the invasive nature of cancer cells, one of the most important and best indicator of them is the markers inside them. One of the most important markers that observed in some types of cancer cells in various parts of the body is the Cytokeratin. Tissue plasminogen activator antigen (TPA) is a Cytokeratin composed of molecules with various molecular weights. The level of TPA serum as associated with cellular growth level and tumorization of cells. In this research, the hybrid of spleen cells in BALB/c female mouse with myeloma cells was conducted with a ratio of 10:1. The resulting monoclonal antibodies were confirmed by SDS-PAGE and western blot. Protein G chromatography was utilized to purify monoclonal antibodies. The results for determining isotypes showed IgM and IgG classes. The titer of the antibody obtained from various clones was capable of identifying Cytokeratin antigen with a dilution of 1/10000. The resulting antibodies were finally confirmed by western blot and all the 5 resulting monoclonal antibodies were capable of identifying a 48 kDa protein. The results indicate that with the help of TPA marker and the monoclonal antibodies produced against them, this marker can be recognized quickly with great accuracy in suspicious cases of cancer. Thus, appropriate measures will be taken to prevent and fight off its probable side effects. This factor can be further used to build a diagonal kit with high sensitivity.

Keywords: Antibody, Monoclonal, Cytokeratin 18, ELISA, Cancer.

INTRODUCTION

Cancer is a disease in which the cells lose their ability to naturally and regularly grow and divide and this will result in the damage of healthy cells as well. Considering the importance of tumor proliferation and the fact that one of the most important indicators of tumor cells is their invasive nature, markers contributing to cell proliferation can clinically inform and predict such issues. One of the factors which predicts the process of cellular invasion is Cytokeratin. Cytokeratins can reflect the proliferating behaviors of tumor cells[1-3].

Cytokeratin is one of the most important Cytoskeletal structures containing composed of 20 separate Polypeptides divided into class I and II [4]. TPA is one of the several types of Cytokeratins which is, in fact, a heterogeneous composed of molecules with the molecular weight ranging from 20 to 68 kDa [5].

The researches conducted in this field confirm a relationship between Cytokeratin tumor markers and Epithelial carcinomas such as breast [6], lung [7], large intestine [8], stomach [9], cervix [10], and bladder [11] cancers. TPA is a valuable marker used to diagnose lung cancer [12]. The general sensitivity of TPA serum assessment in the diagnosis of lung cancer is around 70% and its specificity is 95%. However, the sensitivity of this test for Non-Small Cell Lung Cancer is around 80% [13].

The qualitative evaluation of cancer markers through immunity measurement techniques can be achieved by using polyclonal and monoclonal antibodies. Tumors are identified through expression of antigens on cancer cells and they are diagnosed using special monoclonal antibodies against Epitopes. Monoclonal antibodies are a group of sensitive and precise detectors for this TPA marker in blood and other fluids in the body [14, 15].

Maulard et al. studied histological polypeptide antigen as a marker of bladder cancer among 167 patients. The density of TPA serum prior to and after treatment was measured in them. The results indicated a direct relationship between TPA and the course of the disease. The level of this protein among the people who had undergone treatments is much less than the level observed in those not undertaking treatment. The results indicate that this serum TPA can be considered as factor dependent upon survival [16]. Another research conducted by Pfaff et al. showed that human monoclonal antibodies are produced to recognize TPA antigen indicator in patients suffering from stomach cancer. This antibody was capable of recognizing 45 kDa Cytokeratin 8 antigen through Immunoblotting [15].

The present research seeks to gain monoclonal antibody and purify it against Cytokeratin 18 antigen.

MATERIALS AND METHODS

Procuring antigen and creating immunity in mouse

To create immunity, 5 BALB/c mice (6 to 7 weeks) were immuned 3 times using 5 microgram of human Cytokeratin 18 protein (MyBioSource, USA) prepared in phosphate buffered saline (PBS). The process of creating immunity lasted for 10 days during which the first and second injections were conducted in intraperitoneal way, while the third injection was intravenous. The quantities used to immune mice in the first, second and third injections were 1/500, 1/1000 and 1/5000. The mice in which the antibody titer after the second injection was more than 10^7 were selected and their spleens were removed and used for fusion [17].

The cell line

About 5×10^5 Sp2.0-Ag14 Myeloma cells in a complete cultivation environment of 15 ml composed of RPMI containing 10% FBS, penicillin antibiotics ($100\mu g/ml$) and L. Glutamine was poured in a cultivation flask of 75 cm^2 and then it was exposed to a temperature of 37 degrees centigrade with 5% CO_2. The new cultivation environment was added to the cells every three days.

Fusion of Spleen cells with Myeloma cells

Spleen cells (10^8) were mixed with Myeloma cells (2×10^6) with a ratio of 5:1 and 1 milliliter poly ethylene Glycol was added to the mixture. 1 milliliter RPMI environment was added to it and the process of dilution with PRMI environment was undertaken twice with quantities of 5 and 10 milliliter. Immediately after fusion in 96-alveolate plates, some 200 μl cellular hybrid was added to each sink. The resulting mixture was stored in HAT cultivation environment for 14 days in a temperature of 37 degrees centigrade with 5% CO_2.

After heating them for 14 days, the clones were studied using reverse microscope. Having confirmed hybrid clones for them, ELISA assessment was conducted for them [17].

Determining the isotype of the antibody

3 microgram antigen was added to each 96-alveolate sink. Then BSA 1% solution was added to each sink. 100 microliter hybrid cell with a dilution of 1/5000 was prepared in PBS buffer and added to each sink. Next, 100 microliter of various kit antiserums and 100 microliter of secondary antibodies containing HPR were added to each sink with dilutions of 1/1000 and 1/100000. Color change in the wave length of 450 nm was measured by adding TMB and 1 H_2SO_4 normal substrates.

Proliferation of a hybrid cell in organisms
The BALB/c female rat (6 to 7 months) which was injected in her Peritoneum with 0.5 ml 10 days before was injected with 2×10^6 hybrid cells. 14 days later, 5 ml of the Peritoneum liquid was extracted and centrifuged ($1500 \times g$) for 10 minutes and upper non-oily liquid was separated from it.

Western blot
SDS-PAGE electrophoresis of 2 micrograms of Cytokeratin protein was conducted in denatured and natural conditions (through heat) in the separating phase of Acrylamide 10% gel (150 v, 500 mA, 60 minutes). Afterwards, thin filter, thick filter, Nitrocellulose paper, gel, thin filter and thick filter were placed respectively (14 V, 60 minutes).

Then, nitrocellulose paper was placed in Bovine Serum Albumin (BSA 1%) for one hour and it was rinsed three times by TBST buffer. Nitrocellulose papers were exposed to positive clone for 1 hour and then rinsed again. The resulting strips were then placed on nitrocellulose papers inside Conjugated antibodies (HPR) with a dilution of 1/4000 for 1 hour. Finally, DAB substrate solution was added to them [18].

Purification by Protein G chromatography
Condensation of the antibody was accomplished through ammonium sulfate method and dialysis of the sediments. Purification was accomplished through chromatography with protein G. 0.5 gram protein G was poured inside the tube and the tube containing protein G was rinsed several times by PBS. Antibody which was condensed previously was added to the tube and it was rinsed with the connecting buffer (3.153 gram Tris-HCl, 2.52 gram NaCl in 200 milliliter of distilled water, pH=2.7) several times. Next, the separating buffer (0.75 gram Glycine, 100 milliliter distilled water, pH=2.7) was added and the results were studied in the wavelength of 280 nm [19].

RESULTS

Preparing antigen and creating immunity in mouse
Antibody titer determination was conducted in mice BALB/c, M_1 up to M_5. The first injection was conducted with complete adjuvant, the second one was conducted with non-complete adjuvant and the third injection was done without any adjuvant. The results of ELISA and observations of antibody titer in wavelength 450nm is shown in figure 1.

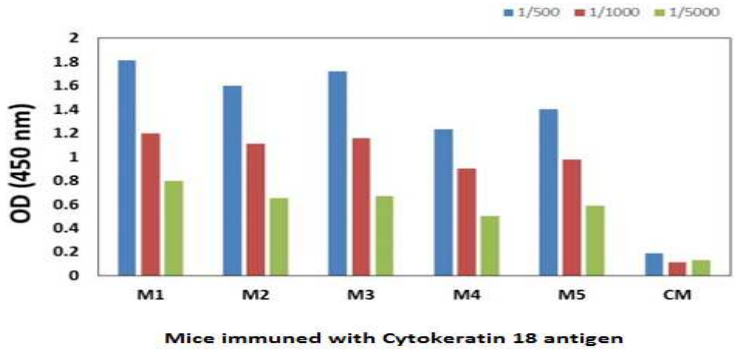

Chart 1. determination of antibody titer of the serum of B ALB/c mice injected with human Cytokeratin 18. Mice M_1 to M_5 were injected twice each time with 5 microgram Cytokeratin antigen. CM as the negative control in mice is shown before injection

Fusion of Spleen cells with Myeloma cells
Determining the isotype of the antibody
The analysis of class 5 of monoclonal antibody was conducted by isotyping kit through ELISA method. In this experiment, P3-D8-s hybrid had the subclass IgGl and 4 other hybrids belonged to IgM class. Table 1 presents the results achieved through the determination of class 5 of monoclonal antibody with isotyping kit.

Table 1: the results achieved through determination of monoclonal antibody by isotyping kit

hybrid name (monoclonal antibody)	antibody class
P1-F2-s	IgM
P3-G1-s	IgM
P3-D8-s	IgG1
P4-C3-s	IgM
P5-E11-s	IgM

Proliferation of a hybrid cell in organisms

To achieve high levels of antibody, the resulting hybrid was injected to the Peritoneum of BALB/c mouse. It was then studied with ELISA to check the existence of high levels of monoclonal antibody after it had been centrifuged. As the results indicate, various clones up to a dilution of 1/10000 are capable of diagnosing Cytokeratin antigen. Table 2 represents the results of the ELISA of various clones.

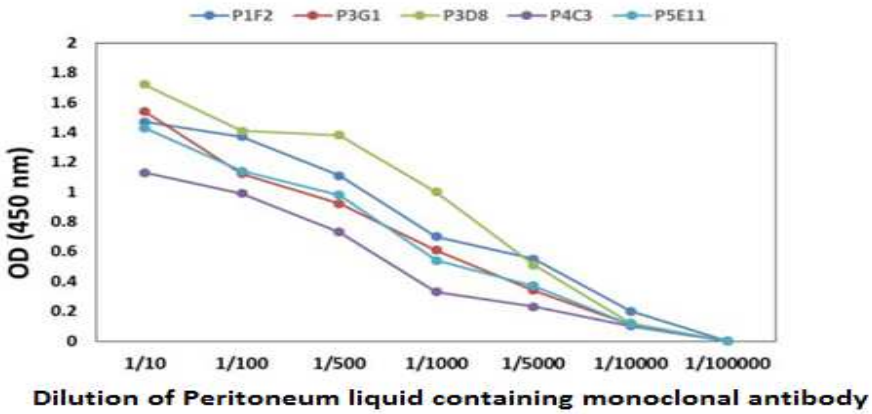

Chart 2. Reaction of various dilutions of Peritoneum liquid containing monoclonal antibody with Cytokeratin antigen

Western blot

SDS-PAGE and western blot were used to diagnose the exclusive antigen with which monoclonal antibodies react. Figure 1 represents the results achieved through the 10% Electrophoresis of Acrylamide gel and gel coloring through kumasi blue.

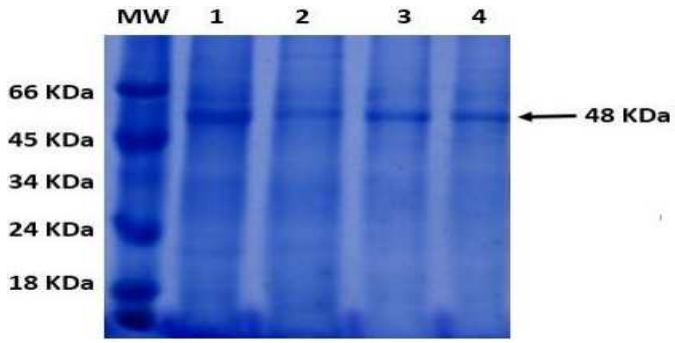

Figure 1: 10% acrylamide gel through kumasi blue MW, 1, 2, 3, and 4 columns represent standard molecules and molecules with 5, 3, 2, and 1 microgram densities of Cytokeratin antigen

Western blot was utilized to observe the protein identifiable by monoclonal antibody and 5 monoclonal antibodies and a 48 kDa protein were diagnosed. Figure 2 represents the results achieved through the western blot of 5 hybrids achieved in diagnosis of Cytokeratin antigen.

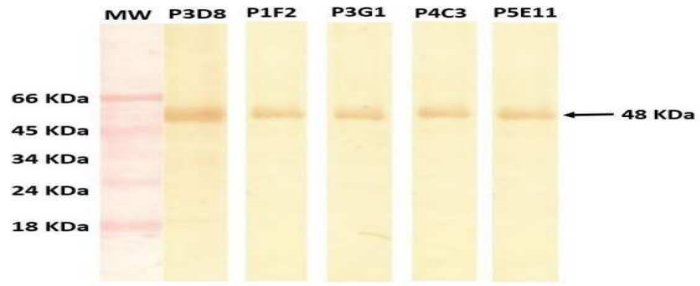

Figure 2: the results attained through the western blot of 5 monoclonal antibodies in diagnosis of Cytokeratin which identify all the subclasses of a 48 kDa protein

Purification by Protein G chromatography

10 milliliter of the Peritoneal fluid condensed from clon P3D8 were collected in different tubes after they had been added to the column and their OD was measured in 280 nm wavelength. Fractions 5 to 9 which contained IgGl were mixed with one another and their density was measured through Bradford method where the amount of IgGl was equal to 150 micrograms in each milliliter of the initial Peritoneal fluid. Chart 3 represents the absorption of the fractions separated from protein G in 280 nm.

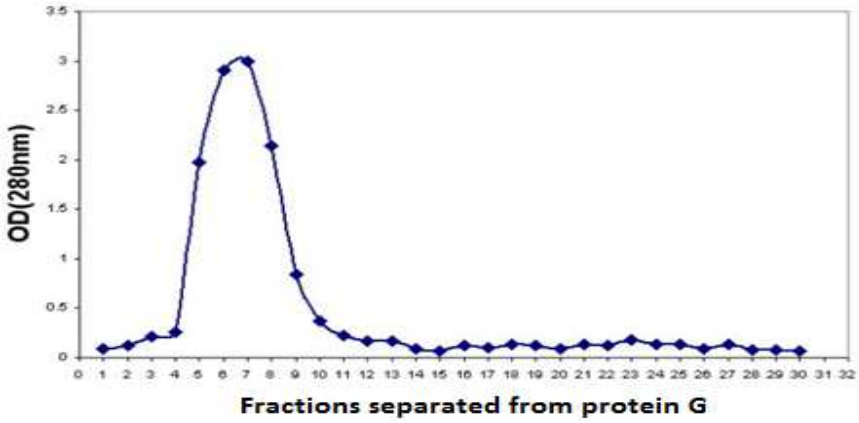

Chart 3: purification of IgG1 by protein G in P3D8 clone

DISCUSSION

One of the main problems in treating the cancer is the quick and timely diagnosis of it. Thus we will need effective and appropriate methods to control and treat this disease. One of the methods for the quick and precise diagnosis of cancer is using appropriate diagnosis markers [20]. Various studies have been conducted about the use of monoclonal antibodies in diagnosis of cancer markers.

Human monoclonal antibody against the cancer marker Cytokeratin 18 is developed in people with stomach cancer. Antibodies produced with IgM class are capable of diagnosing a protein with the weight of 45 kDa in people with stomach cancer [21]. The level of Cytokeratin in the serum of the patients with breast cancer was studied using monoclonal antibody. A direct relationship was shown to exist between the rise in Cytokeratin level and the course of disease. Thus, this protein can be utilized as a diagonal marker in order to diagnose breast cancer [22]. Another research measured the level of TPA using monoclonal antibody on 154 patients with bladder cancer. A direct relationship was observed between this marker and the course of disease. The serum level of this marker increases in people whose cancer is in chronic stages [23].

Tumor with the expression of antigens on cancer cells is identifiable and diagnosis is accomplished with the special monoclonal antibodies against epitopes. Monoclonal antibody is an accurate and sensitive identifier for this marker in blood and other fluids of the body. Human monoclonal antibody is produced in people with stomach cancer to identify TPA antigen indicator. This antibody was capable of identifying the 45 kDa Cytokeratin 8 antigen during

the immuno blotting. Cytokeratin 8 is a marker released by tumor cells during meiosis, secretion and death of a cell [24].

In this research, fusion of spleen cells with myeloma was utilized with a ration of 10:1 and 437 clones were observed totally. 15 clones were observed as positive ones while, finally, 5 clones were left. Having determined the class of various clones, it turned out that one clone had the subclass IgGl and the other 4 clones had isotope IgM. SDS-PAGE and western blot were utilized to determine the antigen recognized by the produced antibody and all these methods identified a 48 kDa molecule.

Considering the fact that measuring the level of special cancer markers is of great importance in diagnosis of the course of the disease and progress of cancer, a standard and precise diagnosis method is required. Keeping in mind the diagnosis accuracy of monoclonal antibody, this diagnosis method can be designed based on ELISA technique. Thus, the monoclonal antibody produced against purified 48 kDa protein can be used as an appropriate candidate to build a diagnosis kit.

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