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PURIFICATION OF IGM MONOCLONAL ANTIBODY FROM ASCITES FLUIDS BY USING FAST PROTEIN LIQUID CHROMATOGRAPHY

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ABSTRACT

Hybridoma clone C3A8 was established as a result of fusion between the lymphocytes of Balb/c mice immunized with the MCF-7 breast carcinoma cell line and Sp2/0-Ag14 myeloma cells. The clone was secreted the monoclonal antibodies (Mab) either in culture supernatant or ascites fluid and still have a contaminants which need to be purify in order to get the desired antibody. The main objective of this study is to purify the Mab. The monoclonal antibodies were purified by using HiTrap IgM Purification column and Fast Protein Liquid Chromatography (FPLC). The flow rate for FPLC system was 1 ml/min and 0.3 bar pressures which successfully separated IgM in crude monoclonal antibodies. Before purification process, the recloning of hybridoma cells by limiting dilutions was carried out in this study and it showed the clone C3A8 secreted IgM monoclonal antibody with kappa light chain. The purified IgM was analyzed using sodium dodecyl sulphate polyalcrylamide gel electrophoresis (SDS-PAGE) indicated that purified IgM had 55 kDa of heavy chain and 27 kDa of light chain. Screening by cell-ELISA showed the purified Mab C3A8 reacted strongly with breast cancer cells (MCF7) and colon cancer cells (HT29). Through immunofluorescence staining, the antigen was detected to be located in the cytoplasm of MCF7 and HT29 cell lines but there were no positive staining detected on cervical cancer (HeLa) and fibroblast normal cells (3T3). The purified Mab was found to react specifically against a 55 kDa protein that was present in the extract of MCF7 and HT29 cell lines when immunoblotting was carried out. All the results mentioned above, suggest that the purified Mab C3A8 could be detected in breast and colon cancers cells.

Keywords: hybridoma, monoclonal antibody, FPLC, cell- ELISA, HiTrap IgM.

INTRODUCTION

Cancer or tumor is the universal name for a group of more than 200 diseases in which the cells in a part of the body will start uncontrolled growth. Cancer cells can break away from this original mass of cells, travel through the blood and lymph systems, and lodge in other organs where they can again repeat the uncontrolled growth cycle. Normal human body cells grow, divide, and die in an orderly trend and during the early years of a person's life; normal cells divide more rapidly until the person becomes an adult. After that, cells in most parts of the body divide only to substitute useless or dying cells and to repair injuries (Elima *et al*., 2012).

Base from Second Report of National Registry a total of 21,464 cancer cases were diagnosed among 9,400 males and 12, 064 females Malaysians in Peninsular Malaysia in the year 2003 (NCR, 2003). From all of the a variety of cancers, breast cancer was the most common cancer that caused death in all ethnic groups and all age groups among women from the age of 15 years. Chinese women had the highest incidence in breast cancer, followed by Malay women and Indian women. Meanwhile colon cancer is the commonest cancer among men and the third most common cancer in women. Chinese had a higher incidence of colon cancers than the other races. Comparing the crude rates between Chinese and Malays, Chinese had more than 5.1 times the incidence of male colon cancer, and 4.6 times the incidence of female colon cancer (NCR, 2003).

Breast tumor is the tumor that begins from breast tissue. Most commonly it is from the cells of the inner lining of milk ducts or the lobules that supply the ducts with milk (Barbara, 2001). Cancers originating from ducts are known as ductal carcinomas and those originating from lobules are known as lobular carcinomas. Molecular events that contribute to the increased motility of tumor cells have become important for understanding tumor metastasis as well as for targets of potential therapeutic intervention in human cancers. Human breast cancer MCF-7 is estrogen-receptor positive epithelial tumor cell line. It is usually used as an experimental cell model because of its poorly invasive capacity (Meng *et al*., 2000, Sood *et al*., 2006). This cell model is helpful for exploring genes aberrantly expressed in tumor cells that contribute to tumor metastasis (Yuecheng *et al*., 2006).

Monoclonal antibody (Mab) played an important role; it may substitute the other treatment method such as surgery, chemotherapy and radiation therapy. A stable hybridoma clone C3A8 was established by Ali *et al*. (1996) as a result of fusion between the lymphocytes of Balb/c mice sensitized with the MCF-7 breast carcinoma cell line and Sp2/0-Ag14 myeloma cells. The clone showed 100% positive towards breast cancer cells line

after five limiting dilutions and secreted IgM monoclonal antibody with kappa light chain. The hybridoma was capable of secreting monoclonal antibody in serum free medium and ascitic fluid. Mab produced by C3A8 reacted very strongly to the human breast cancer cell lines MCF-7 and T47-D but showed negligible reactivity against other human cancer cell lines. Results of immunohistochemical test of Mab C3A8 with various human tissues using immunoperoxidase method showed the Mab reacted to ten cases of invasive ductal breast carcinoma, two cases each of lobular breast carcinoma and fibroadenoma (Ainul Fajariah *et al*., 2007). No positive signal was observed on tissue sections of benign phyllodes tumor and ductal papilloma of benign breast, uterine leiomyoma and carcinoma, intestinal metaplasia cervical carcinoma, tonsillitis and neurofibroma. The reactivity of Mab C3A8 towards MCF-7 cells was markedly reduced when the cells were treated with trypsin but not with neuraminidase or periodate. Immunohistological studies showed that the Mab reacted to lobular breast and fibroadenoma cancer tissues at the cytoplasmic region. Even though after obtaining either a media sample of cultured hybridomas or a sample of ascites fluid, they still have contaminants which need to be purified to get the desired antibodies. The contaminants in the cell culture sample would consist mostly of media components such as growth factors, hormones, and transferrins. In contrast, the in vivo sample is likely to have its host antibodies, proteases, nucleases, nucleic acids, and viruses. In both cases, other secretions by the hybridomas such as cytokines may be present. Moreover, there may also be bacterial contamination and, consequently, endotoxins that are secreted by the bacteria. The traditional approaches for purification of IgM have been based on a combination of methods such as precipitation, chromatography and electrophoresis. These methods, however, suffer from several serious drawbacks. These techniques are non-specific in their binding and thus vulnerable to cross-contaminations. A one stage separation approach has been inadequate in providing highly pure IgM. On the other hand, affinity chromatography which is based on biospecific ligands, involves specific, reversible and non-covalent interactions between an immobilized ligand and the target protein for purification. This technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure IgM present at low concentration in large volumes of crude sample and also to remove specific contaminants.

MATERIALS AND METHODS

A stable hybridoma clone C3A8 was previously established by Ali *et al*. (1996) as a result of fusion between lymphocytes of Balb/c mice sensitized with the MCF-7 breast carcinoma cell line and Sp2/0-Ag14 myeloma cells. This hybridoma was revitalized from liquid nitrogen storage (Animal Tissue Culture Lab, Biotech 3, Faculty of Biotechnology and Biomolecular Sciences, UPM) by thawing the cells at 37°C and transferred into a culture flask with the cell growth media [RPMI-1640 medium, (Sigma USA) added with 10% (v/v) of fetal bovine serum (FBS) (GIBCO, USA) and 1% (v/v) of 100 unit/ml of penicillin and streptomycin (GIBCO, USA)]. After reaching confluent the cells were sub-cultured by transferring one ml cell culture into 9 ml of fresh growth media or the culture was split equally with fresh growth medium. The cells were maintained in an incubator at 37°C supplied with 5% (v/v) of CO2 at animal tissue culture laboratory Universiti Sultan Zainal Abidin.

MCF-7 (breast carcinoma); HT-29 (colon carcinoma) and HeLa (cervical carcinoma) were obtained from the American Type Culture Collections (ATCC). The cells were grown in cell growth media and maintained at 37°C incubator supplied with 5% (v/v) of CO2. The anchorage dependent cells have to be detached from the surface before they can be subcultured. The cells were detached by adding 100 ul of trypsin for 5 minutes to break down the proteins responsible for surface adherence. The detached cells were then resuspended with 200 ul growth medium and allowed to settle back onto their growth surface. The detached cells were then centrifuged at 1300 RPM for 10 minutes. The pellet that was obtained after centrifugation was further grown in new flasks with fresh growth media.

Cloning of Hybridoma Cells C3A8 by Limiting Dilutions

One hundred micro liter of hybridoma cells C3A8 that reached confluent stages were transfer into centrifuge tube contained 12 ml of RPMI-1640 media that supplied with 5% FBS. The cells concentration was counted by using a haemocytometer. The suspension hybridoma cells were further diluted in 20 ml RPMI-1640 that supplied with 20% FBS to give five cells per mL (one cells / 200 µL). From the dilution, 200 µL of the suspension was pipetted into each well of 96-well plates (Nunc, Denmark). The plates then were maintained at 37°C incubator supply with 5% (v/v) CO2. The wells with single colony were marked and the supernatant was tested for the presence of Mab using ELISA technique. The cells from the well that gave higher OD value were transferred into 24-well plate and 6-well plate that contained RPMI-1640 supplied with 20% FBS. The cells were then transferred into 25 cm2 tissue culture flask when they reached confluency.

Antibody Screening by Cell ELISA

The presence of monoclonal antibody in the hybridomas supernatant and ascites fluid were assayed by cell-ELISA method as described by Ali *et al*., (1996). The 70% of confluent MCF7, HT29 and HeLa cell at 1x105 cells/ml was coated at 200 uL in 96 wells plate

(Nunc, Denmark) and was incubated overnight in 37°C incubator. Media from cells that reached the confluent was discarded and the monolayer cells were washed with phosphate buffer saline (PBS) three times before being fixed with 100 µL of 0.06% glutaraldehyde (Sigma USA) for 20 minutes at room temperature. After the glutaraldehyde was discarded and washed with PBS, 300 uL of 1% (w/v) of bovine serum albumin (BSA) was added into each well as a blocking solution and the plate was incubated again at 37°C for one hour. The plate was washed with PBS for three times before a volume of a 100 uL of hybridoma supernatants or ascites fluid from each dilutions were added into a well as primary antibody. After incubation at 37°C for another one hour, the wells were washed three times with PBS and 100 uL of goat anti mouse IgM-HRP (KPL, USA) (1:1000) was pipetted into each well as a secondary antibody. The plate was incubated at 37°C for one hour. Eventually, the well was washed with PBS and 100 uL of ready to use ABTS substrate (KPL, USA) was added in the dark into each well. The plate than was incubate at room temperature as long as 30 minute. One hundred uL of 1% (w/v) sodium dodecylsulphate (SDS) was added to each well to prevent the enzymatic reaction. The plate was read at 411 nm by using the ELISA Plate Reader to measure its OD value.

Propagation of Hybridoma Cells in Balb/c Mice (In-Vivo) for Ascites Fluids Production

The balb/c mice 6 weeks old were injected intraperitonealy (ip) with 0.5 ml of pristane (2, 6, 10, 14-tetramethyldecanoic acid) (Sigma USA). Balb/c mice were used since most mouse hybridomas are derived from myeloma cells and spleen cells originating from this mouse line (Brodeur *et al*., 1985). The needle (Terumo, TOKYO) size for intraperitoneal injection in the mice was 26 ½ gauge. The mice were swab with alcohol swab that was saturated with 70% isopropyl alcohol prior injection. The pristane would acts as irritants to the Balb/c mice which respond by secreting nutrients and recruiting monocyte and lymphoid cells in the area. This creates a good condition for the hybridoma cells growth. After two weeks, 0.5 ml of 3.56 ×106 cells/ml hybridoma cells C3A8 in PBS was injected into the pristane treated Balb/c mice. Before the injection, the hybridoma cells were spun at 1000 rpm in a 15 ml centrifuge tube; the supernatant was discarded and gently resuspended in PBS. The abdominal distension of mice was observed daily. Ascites fluids are taken about 7-14 days to aspirate after the cell injections. Aseptic technique was used in collecting of ascites fluid. The smallest sterile needle 18 gauge was used to allow a good flow of ascites fluid. The needle was inserted in the lower left quadrant of the mice abdomen opposite the site of inoculation. The needle was rotated and the depth of insertion was adjusted to allow the optimizing rate of collection. The ascites fluid was allowed to drip from the needle hub into the 50 ml sterile centrifuge tube. After the collection of ascites fluid in the sterile centrifuge tube, the ascites fluid was let to stand at room temperature for 30 minutes. The ascites fluid then was centrifuged at 10 000 x g for 30 minutes to separate the serum from other blood components and the straw-colored fluid was transferred into other sterile centrifuge tubes with a sterile Pasteur pipette. The ascites fluids then were filtered through 0.45 µm membrane and were stored at -80°C till the time of purification. This animal experiment was approved by the animal research committee Universiti Sultan Zainal Abidin with certificate number UDM/AEC02/09-02.

Purification of Monoclonal antibody with FPLC using HiTrap IgM Purification Column

The chromatographic experiments were carried out using an ÄKTA Explore 100 fast-protein liquid chromatography (FPLC) system (GE Healthcare) and HiTrap IgM Purification Columns. This system was equipped with multiple automated features, including the capability of collecting peaks with various information, buffer and pH scouting, and shaped elution gradients. The accompaniment with an IV-980 valve provides the capability of running additional sequential elution gradients. The wire loop that was used in this experiment was 100 µl sized. Prior to each chromatographic run the column was equilibrated with the respective binding buffer (Raja Ghosh, 2001) with 100 µl of crude sample that was diluted in 900 µl of binding buffer before being injected 500 µl to the column. Prior injection to chromatography sample was clarified through centrifugation and filtered with syringe filter 0.45 µm. The sample was then precipitated by the addition of an equal volume of 0.8 M of saturated ammonium sulfate solution. The mixtures of precipitated Mabs were spun and filtered again as previously. The precipitate must be freshly prepared. The system pump and column were washed first with distilled water followed with 20% of ethanol prior to sample injection. Both pump A and B that contained binding buffer and elution buffer were switch on. The chromatograph process was started with the flow rate 1 ml/min and pressure of 0.3 bars. All of chromatography experiments were conducted at room temperature and 3 ml fractions was collected. Peak of each fraction were pooled and analyzed with a few assays. The protein band from each peak was visualized using silver staining protocol.

SDS-PAGE

Polyalcrylamide gel electropherosis (SDS-PAGE) was performed using a protein electrophoresis apparatus. The apparatus was assembled according to the instructions by the manufacturer. The separating gel of 12% was prepared in a beaker as much as 10 ml. The components used for separating gel were 30% acryl amide mix, 1.5 M Tris (pH 8.8), 10% SDS (Sodium dodecyl sulphate), 10% APS (Ammonium per sulfate) and TEMED. The mixture of separating gel was rapidly

loaded into the space between the glass plates until the mixture achieved at stacking gel level. Several drops of distilled water were added to remove unnecessary bubbles that trapped in the gel and also unpolymerized acrylamide. A few minutes later, the gel was polymerized and the distilled water was drained out. Next, 5% of stacking gel as much as 6 ml was prepared. The components used was the same as separating gel but only the Tris (pH 8.8) was changed with Tris (pH 6.8). The stacking gel was loaded directly to the surface of the polymerized separating gel. Instantaneously, a clean Teflon comb was inserted into the stacking gel solution carefully to avoid any bubbles to be trapped. After the gel polymerized, the samples in loading dyes (1:1) were prepared by heating at 100°C for 6 minutes to denature the proteins. After the polymerization was completed, the comb was removed and the gel was inserted in the protein electrophoresis apparatus. The running buffer (Tris-glycine electrophoresis) was added to the top and the bottom of reservoirs in apparatus. A volume of 10 µl of proteins was loaded into the wells. Voltage of 110 V was applied until the separation was completed. The gel was taken out and the stacking gel was cut. It was then stained by using silver staining methods to visualize the desired band of protein.

Immunofluorescence

The cell lines used for immunohistochemistry studies were MCF7 , HT29, HeLa and 3T3 were grown in 8 chamber tissue culture microscope slides (Nunc) until the cell become 80% confluent. The media was discarded and the anchorage dependent cells were washed gently with PBS pH 7.4 and fixed with the cold methanol (-20°C) for 20 minutes at room temperature. After fixation processed, the cold methanol was discarded and washed with PBS before incubate with 200 uL of bovine serum albumin (BSA) in each well as blocking solution at room temperature for 1 hour. The purified IgM dilution (1:100 in PBS) were added to each well and incubated at room temperature for three hours to allow the reactivity. The adherent cells were wash again and 200 µL of fluorescein-isothiocynate (FITC) labeled goat anti mouse IgM (chemicon, USA) (1:50 in PBS) were added and incubate for 1 hour at room temperature. The 8-chamber tissue culture microscope slides were observed under an inverted fluorescence microscope.

Western Blotting

The cancerous cell MCF-7, HT29 and normal cell 3T3 (normal mouse fibroblast cells) already detached with trypsin were first lysed with 700 µl lysis buffers in micro centrifuge tubes. The cells were lysed on a roller overnight to mix the solution. After 24 hr, the lysed cell was spun at 10,000 g for 20 minutes. The supernatant was discarded into a new micro centrifuge tube and kept at -20°C before further experiment. The lysed cell was analyzed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis gel was transferred to PVDF membrane by using electro blotter at 15 V for 3 hours. The positive band from PVDF membrane was detected following the instruction manual included in the Western Breeze Chromogenic Immunodetection Kit (Invitrogen, USA) which contains complete, optimized, ready to use or ready to dilute reagents. The membrane was blocked with blocking buffer for one hour to avoid non-specific binding. Ascites fluid containing monoclonal antibody C3A8 already purified at 1:10 dilution was used to incubate the membrane for one hour. The membrane was washed with washing buffer as before. After incubated with secondary antibody goat-antimouse conjugated alkaline phospatase solution (Western Breeze, USA) the membrane was washed again. Immunoreactive bands were detected by chromogenic substrate (NBT substrate for alkaline phosphatase) until purple to dark blue bands developed on the membrane (1-60 minutes).

RESULTS AND DISCUSSIONS

Two techniques were described to produce monoclonal antibodies. The monoclonal antibodies either can be obtained from the hybridoma culture supernatant in tissue culture flask (in vitro) or by growing the hybridoma cells into Balb/c mice peritoneal cavity (in vivo). Previous study from Mahana and Paraf, 1993 reported that producing ascites fluid method may achieve a high yield of monoclonal antibody titers. By using a suitable strain of mice and adjuvant, it can generate high serum titers with high titer range between 1:1000-1:20,000 (Susan Ker-hwa Ou, 1993). Production of ascites fluid was done to achieve the objectives of this study which was to produce high titer of monoclonal antibody.

Previous procedures by Amyx 1987, for producing polyclonal antibodies in ascites fluid occupied pretreatment of mice with pristane (2, 6, 10, 14-tetramethylpentadecane). This chemical is thought to raise ascites fluid volume by inducing granulomatous reactions that interfaced with peritoneal fluid drainage. On the other hand, large volumes of pristane injections have been associated with weight loss, a hunched appearance and a lack of activity in mice inoculated intraperitoneal (Amyx, 1987). In this study, male and female Balb/c mice aged 6 weeks were primed with 0.5 ml pristane as long as 14 days prior the injected with 3.56 x 106 cells/ml of hybridoma C3A8 cells. Brodeur *et al*., (1984) reported that 0.5 ml pristane was an important parameter that affected the production of ascites fluids. The mice inoculated with cells then were checked daily for the swelling of their peritoneal cavities. The most swollen rapidly appeared in male Balb/c mice. The previous study reported that the period of secretion, the time of survival and monoclonal antibody production were significantly better in males (Brodeur *et al*., 1984). After 21 days of cell inoculation ascites fluids were ready

to harvest from every mouse. There were 4ml of ascites fluid was collected from each mice. Antibody titer was determined by cell ELISA method which showed the OD value (411 nm) for ascites fluids were higher compared with antibody from culture supernatant. These results showed, for the reason that of the higher antibody concentration produced by productions of monoclonal antibody in ascites fluids compared with culture supernatant. Figure-1 showed the graph of OD value comparison for antibody titer with serial dilution until 5 times, between supernatant and ascites fluids.

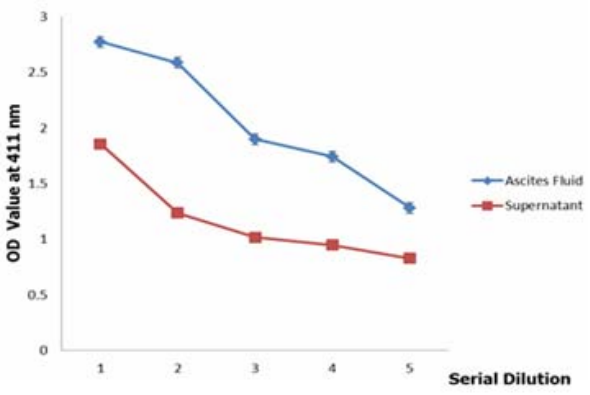

Figure-1. Comparison of the reaction Mab towards breast cancer cells (MCF7) between crude ascitic fluid and supernatant. Noted: media without serum as negative control; the OD value: 0.37. (The serial dilution was done at antibody dilution 1:2).

Purification of IgM Monoclonal Antibody

Antibodies are imperative tools that are being utilized by many of researchers in their studiesand led to many therapeutic advances. Immunoglobulin commonly used in diagnostics applications was mammalian sera that represented a significant and economical source (Gathumbi *et al*., 2001). The desired antibodies must be purified after obtaining the Mab sample either from a cultured supernatant (in vitro) or a sample of ascitic fluid (in vivo) before it can be used as diagnostics tool in order to make it more efficient in binding with the antigen. Supernatant from cell culture samples commonly consist of biological substances from media such as serum, hormones and transferrins. In contrast, ascitic fluid probably has host antibodies, nucleic acids, nucleases and viruses. A combination of methods such as precipitation, chromatography and electrophoresis were a traditional advanced for purification of IgM. Although, these techniques were non-specific in their binding and thus vulnerable to cross-contaminations. A one stage separation approach has been inadequate in providing highly pure IgM. The multi-step procedures were currently implemented, which increases the total cost and time for downstream processing. On the other hand, affinity chromatography which is based on biomimetic and biospecific ligands, involves specific, reversible and non-covalent interactions between an immobilized ligand and the target protein for purification. This technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure IgM present at low concentration in large volumes of crude sample and also to remove specific contaminants (Gautam and Kai-Chee, 2010).

There were two molarities of ammonium sulphate in binding buffer used in this study, 0.8 M and 1.2 M. The Figure-2 showed an elution profile peak for ascites fluid samples with molarity of binding buffer ammonium sulphate 1.2 M. Elution profile contained two peaks. The first peak represented a flow through or unbound material, and second peak was bound material, which was the IgM. Those results showed that, the elution profile purification for ascitic fluid was higher than supernatant and the binding buffer molarity was good at 1.2 M for high elution peaks. In order to confirm the validity and purity of eluted material, each elution from every peaks and crude material was subjected to SDS-PAGE analysis. As in Figure-3, the SDS-PAGE analysis of purification of ascites fluid produced by hybridoma clone C3A8 by thiophilic affinity chromatograph using silver staining. From the analysis, lane 3 showed the molecular weight of IgM, for heavy chain was 55 kda and its light chain was 27 kda.

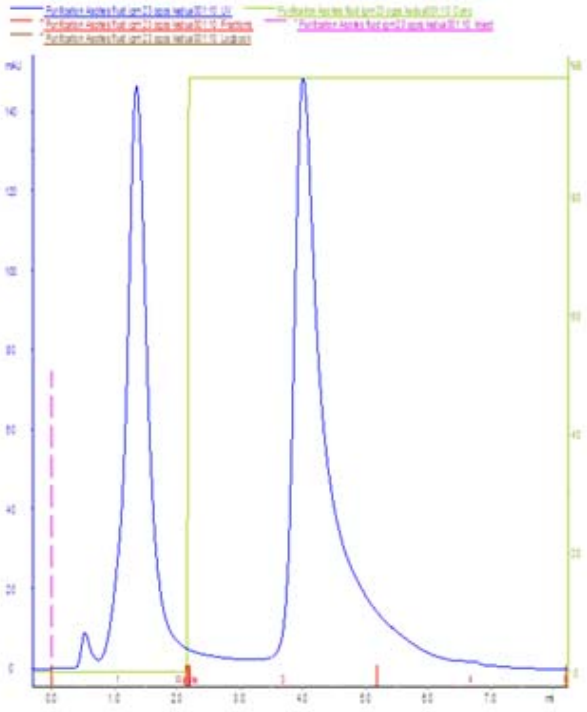

Figure-2. Elution profile obtained from thiophilic affinity chromatography of ascites fluids IgM Monoclonal antibody clone C3A8 samples. First peak = flow-through; second peak = IgM; with flow rate 1 ml/min, pressure 0.3 bar , binding buffer ammonium sulphate concentration 1.2 M, pH 7.5.

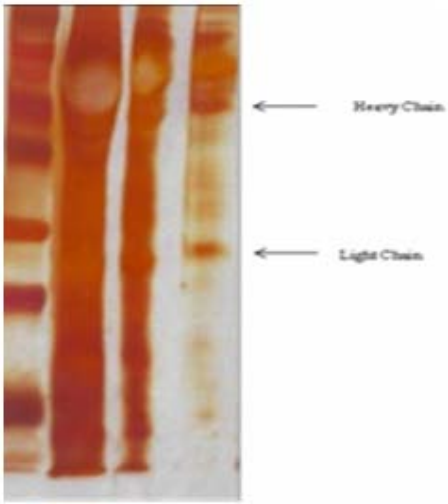

Figure-3. SDS-PAGE analysis of purification of Ascitic fluid produced by hybridoma clone C3A8 by thiophilic affinity chromatograph with 1.2 M binding buffer, using silver staining. Lane M: Protein marker, lane 1: crude ascites fluid, lane 2: flow through first peak, and lane 3: IgM second peak. (Noted: The elution profile from Figure-2).

Immunofluorescence

The method of immunofluorescence is a procedure used for ultraviolet light microscopy with a specific dye that is observed under a fluorescence microscope. Immunofluorescence is commonly used for the study of cell surface antigens. This method was first introduced by Coons *et al*., (1941). There are two types of immunofluorescence; direct and indirect immunofluorescence. In this study, indirect immunofluorescence was used which two antibodies, primary antibodies Mab C3A8 that recognize the target molecule and was binds to it, and secondary antibody that carried the fluorescence, recognized the primary antibody and binds to it.

After purification process of antibody using FPLC was done, the purify monoclonal antibody C3A8 from ascites fluids was test with immunofluorescence technique in order to identify antigens expressed on breast and other cancer cells line. By using dilution at 1:50 of purified antibody the experiment was indicated that a bright of fluorescence labeled on the cytoplasm of breast cancer cells (MCF7) but not on it nucleus as in Figure-4. The same experiment was executed on other cell lines such as colon cancer cells (HT29) and cervical cancer cells (HeLa). The results for cervical cancer cells (HeLa) were negative but for colon cancer (HT29) it also indicated a bright fluorescence on its cytoplasm as in Figure-5. The control experiment also performed on normal mouse fibroblast cells (3T3) as a result it showed a negative result as well. Obviously, the purified Mab had an ability to bind with antigen more than non-purified antibody. As a result, there were more cells that labeled with fluorescence when using purified Mab as the primary antibody.

From previous study by Ainul Fajariah (2007), they confirmed that the monoclonal antibody from crude ascitic fluids gave a positive staining for breast cancer and colon cancer in immunofluorescence experiment with same dilution of antibody solution 1:50. In this study, the method of immunofluorescence was also performed with crude ascites fluids in order to confirm its validity from previous studies. The technique of immunofluorescence allowed the study of the binding of antibody at the single cells and showed the type labeling of positive cells (Price *et al*., 2008) (Boucheix *et al*., 1982). Collinsa (2009) was used immunofluorescence method to detect the subtyped of amyloid in cardiac and renal specimens.

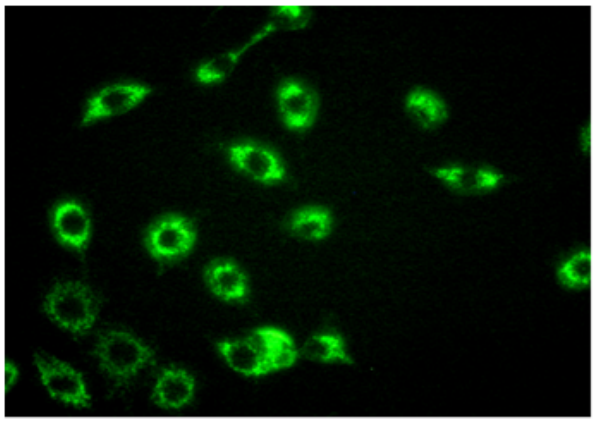

Figure-4. The immunofluorescence reactivity of purified Mab C3A8 (ascites fluids) features on MCF-7 (breast cancer cells) captured under fluorescence microscope (Magnification 400X).

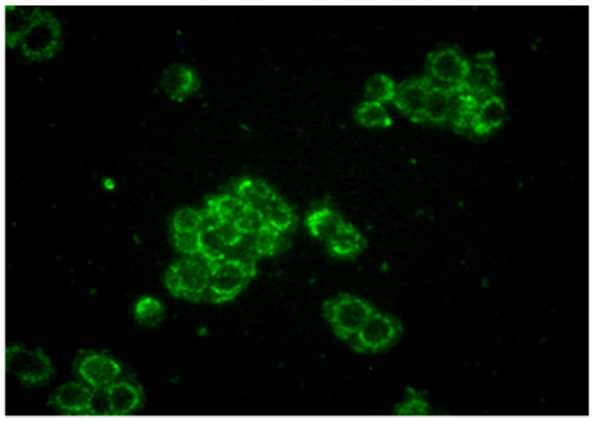

Figure-5. The immunofluorescence reactivity of purified Mab C3A8 (ascites fluids) features on HT29 (colon cancer cells) captured under fluorescence microscope (Magnification 400X).

CONCLUSIONS

The IgM of hybridoma clone C3A8 in ascites and supernatant were successfully purified using prepacked column (HiTrap IgM purification coloum) at 1 ml/min and 3 bar pressure. The elution profiles of purification of Mab C3A8 showed highest peak as it was bounded more when using 1.2 M binding buffer and pH 7.5 compared with 0.8 M binding buffer at the same pH. The purified IgM Mab C3A8 indicated that the heavy chains molecule was 55 kDa and 27 kDa for its light chains when assayed with SDS-PAGE analysis. IgM quantity was more in ascitic fluids compared with supernatant and was quantified using IgM quantification kits. The reactivity of purified Mab C3A8 against different types of cancer cell lines in a few assays was investigated. From the results of this study, it showed that the purified Mab C3A8 was positively bound and reacted with MCF7 (breast cancer cells) and HT29 (colon cancer cells) cells lines through cell-ELISA method. However, when the purified IgM screened by immunofluorescence assay, it showed only positive results with MCF7 and HT29 cell lines but not in HeLa cells. The epitope recognized by purified Mab C3A8 also identified through western blotting assay. The results showed that purified IgM Mab recognized the same molecular weight of protein 55 kDa from the extracts lysis of MCF7 and HT29 cell lines. All the results in this study suggested that the IgM Mab C3A8 can be purified by using Hi-Trap IgM Purification Column and its purity showed positively reacted with breast and colon cancer cell lines. The Mab C3A8 can potentially be used in detection, diagnosis and therapy of breast and colon cancer like others cancer treatment include surgeries, radiotherapies and chemotherapy drugs.

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