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Dot blot assay for the best dilution of Balb/C mouse liver, heart, Lung organs and HT29 protein samples with specific anti bodies

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KEYWORDS

Monoclonal antibodies, Balb/C mouse, mAb IBMR3, Dot blot Assay, BioImaging, HT29 (Human Colorectal) cell line, Spectrophotometer.

A B S T R A C T

The aim of this study for the dot blot assay, was to find the best dilution for antigen and anti-bodies with low concentrations in clear and best background. Using monoclonal IBMR3 as specific primary antibodies in order to recognize specific antigens in the different samples taking from four months Balb/c mouse, heart, liver and Lung organs, in addition of the HT29 Human Colorectal Adenocarcinoma cell line. Using in dot blot assay which is widely use in this filed. Protein extract were extracted from the above samples and used for dot blot assay to widely use in this filed. Protein samples extracts were soaked in small circles of PVDF membrane as antigens with the same amounts of protein samples but the concentration was different, and IBMR3 monoclonal antibodies were added with the same amounts for each disc and incubation as primary antibodies, and then secondary anti bodies (rabbit anti mouse–HRP) were added with the same amounts, but in different concentration to detect the best dilution for antigen and anti-body with low and best background. The result of this research depends on the PVDF disc color in the Mircotiter well, color shows the best color result that gives best low background in mouse samples, the results reading was presented in table 5, best dilution for the antigen in protein samples for the heart was (1/5), liver 1/25, lung 1/25 and HT29 1/ 25. The best dilutions of secondary antibodies were 1/50 with heart, 1/100 with liver, 1/25, 1/50 with lung, and 1/25 with HT29. The results from this study suggest that the IBMR3 antigens are expressed in all investigated, heart, Liver, Lung organs and HT29 Cancer cell line antigens, but the expression pattern varied from organ to another. This might be highly indicative for expression profiles of this antigen. The expression of IBMR3 Ag in cell lines (HT29), this might also be indicative of the fact that IBMR3 antibody can express or recognize the same epitope in different molecules having different relative molecular masses. MAbs IBMR3 may be useful to identify and study the roles of these molecules in normal tissue and cancer cell lines. This may help in future to use IBMR3 MAbs in cancer tissue and cells to identify the molecular weight of each IBMR3 Ag to be indicator for some specific type of cancer, that is, it has potential to be a future cancer marker.

Introduction

Now days the use of monoclonal antibodies has increased by Scientists in laboratory diagnosis and treatment for some diseases.

Monoclonal antibodies (mAb or MOAB) are monospecific antibodies that are indistinguishable because they are raised by

one type of immune cell that are all clones of a single parent cell. IBMR3 is a mAb of IgM isotopes, produced previously by using synthetic peptides corresponding to selected amino acid sequences of the IL-4 receptor molecules [1 & 2]. mAb can only be produced in certain strains of mouse or rat with histo-compatibility plasmacytoma fusion lines [3]. These mice or rat have normal basal levelsof IgM and of IgG isotopes with normal B and T-cell development [4].

MAbis a single type of antibody, produced by B-cell clones of a single hybridoma or single parent cell line. A hybridoma cell line is formed by the fusion between one cell of normal B lymphocyte and a myeloma cell in cultures media i.e. (PEG) (poly ethylene glycol) and HAT (hypoxanthine Aminopterin Thymidine) [5]. Hara and Mat, [3] provided evidences that mAb IBMR3 might recognize the same epitope which is shared by molecules having different molecular masses.

MAb can be generated against most target antigens, purified and split into fragments. MAb has the ability to conjugate with radio nuclides, toxins, enzymes or drugs. By nature, mAb originate from one specific clone with higher specificity, purity, consistency and identify only one epitope of the antigen. However, these antibodies require secondary antibodies which are used in multiple analyses [6]. Such methods are used in laboratories for common techniques in many medical research and diagnostics [5].

MAb are also used in immunotherapeutics because they cooperate with immune system cell molecules to produce anti-tumor responses and can increase the intensity of immune reactions against tumor by Ligand formation with receptors on lymphocytes cells or antigen-presenting cells [7]. The antigen proteins involved in the immune reaction can be separated by the process of electrophoresis according to the molecular weight of their polypeptide chains [8]. To confirm further the antibody specificity for the proteins western blotting is the most ideal technique practiced in Immunology and Microbiology [9].

MAb are potentially capable of multiple functions. Efficacious anticancer mAb must bind to an appropriate antigen in quantities sufficient to mediate a disease-relevant response. The mode of antibody action to destroy any target can be direct via conjugated radioactive isotopes or toxins, or antibody triggered apoptosis or indirect by activation of immune system components or blockade of critical receptors [10]. The main aim of this study was to analyses the antigen specific expression of specific monoclonal IBMR3 antibodies between Balb/c mouse and rating using heart, Lung, Liver and HT29in dot blot assy.HT-29 is a human colorectal adenocarcinoma cell line [11].

Materials and methods

Preparation of lysis buffer (RIPA)

200 µl of (5X Buffer, Tris- EDTA) was mixed with 200 µl of (5X NaCl),and then 200 µl of (5 X SDS Lauryl) were added to the mixture, then 200 µl of (5X, deoxycholic acid) were added to previous buffer then follow with 200 µl of (5X Igepal CA 630) to Mixture, following with 10 µl of protease inhibitor cocktail to get the final volume of 1 ml. The lysis buffer was stored at room temperature.

Note: 1ml of RIPA lysis buffer was used to extract 5-20 mg of ground tissue sample or 10^6 -10^7 cells.

Preparation of HT-29Cell Culture (Human Colorectal Adenocarcinoma) Line

The cells were harvested using cell dissociation solution 3ml / 25 cm² flask or 5 ml / 75 cm² flask for about 15-20 minutes for cells to dissociate and (monitor under the microscope for floating cells). The solution was decanted and the flask was tapped to lose the cells. Then 10 ml PBS sterile solution were added and flushed inside the flask bottom to loosen the cells. The cells were pipetted into a 15 ml sterile falcon tube to spin under 2,000 rpm for 5 minutes and then PBS decanted. The cells were washed two times with PBS and centrifuged then decanted again and the cell was kept frozen at (– 20 to - 30) °C.

Protein extract from HT29 cancer cell line

Frozen sample for HT29 sample was thawed at room temperature before lysis until 4-8 °C. Care was taken to ensure homogeneity of the samples.Then 10^{6} -10^{7} of harvested frozen cells were taken and placed after thawing in 1ml of lysis buffer in an Eppendorftube, incubated for 15 minutes in an orbital shaker at room temperature, ThenThe sample was vortexed for 60s and then centrifuged in a micro centrifuge at 12000 rpm for 10 minutes to pellet the cellular debris. Protein rich supernatant was then removed and pellet decanted. The extracted proteins were used either immediately or kept in ice or stored at -70°C if not used immediately.

Preparation of grinding samples from liver, lung and heartorgans

Materials

The materials and chemical were of analytical grade and includes Balb/c mouse 3 month old, PBS (Calbiochem), liquid nitrogen, scalpel & scissor, petri dish, ethyl alcohol (Sigma) and small test tube.

Preparation

The Balb/c mouse was sacrificed by cervical vertebra dislocation. Incision midline and the abdomen were done; the three organs were removed and transferred each organ in petri dishes separately to cut each sample in suitable size pieces. The samples were labeled and transferred in a cryo vial with 25 µl PBS; the cryo vials then stored in liquid nitrogen for a long time or use after freezing at the same time.

Grinding tissue preparation from frozen sample

Materials

The materials and chemical were of analytical grade and includes mortar and spatula, small test tube, liquid nitrogen, protective gloves and protective eyes.

Method

The organs were from Balb/c mouse (heart, liver, lung) were all ground under liquid nitrogen using a pestle and mortar. The samples were either stored in liquid nitrogen or prepared for treatment in lysis buffer.

Protein extract from organs grounding samples

After thawing the frozen samples of lung liver and heartat room temperature, 1ml cell lysis buffer were added to 5-20 mg of each sample in three Eppendorf tube and incubated for 15 minutes in an orbital shaker at room temperature. All the samples were vortexed for 60s and then centrifuged in a micro centrifuge at 12000 rpm for 10

minutes to pellet the cellular debris. Protein rich supernatant was then removed and pellet decanted. The extracted proteins were used either immediately or kept in ice or stored at -70 °C if not used immediately.

Quantification of protein concentration using Amersham biosciences 2-DQuant Kit

A working color reagent was prepared by mixing 1 part from color reagent B with 100 parts from color reagent A. Each individual assay required 1 ml of working reagent.

Working reagent was stored at 4-8 Cº for up to one week or as long as the optical absorbance (A 480) of the solution remained below 0.025 at 480nm.

A standard curve was prepared according to table 1, using a concentration of 2 mg / ml of standard bovine serum albumin (BSA). Six tubes were prepared for blank without BSA (0), 5 µl, 10 µl, 15 µl, 20 µl, 25 µl of protein.Protein quantity/protein known concentration as: 0µg 10µg 20µg 30 µg 40µg 50µg.

BSA = Standard protein solution

Samples Preparation for Protein Quantification

Determination of Protein in, HT29 (Cell Lines) and Balb/C mouse Samples

15 µl of each sample protein extract of HT29, liver, lung, and heart were placed in an Eppendorf ® tube; the test were done in duplicate.

As in table 2, 50µl precipitant was added to each tube (including the standard curve tube), vortexed briefly and incubated for 2-3 min at room temperature. 500µl co-precipitant was added to each tube and briefly vortexed. The tubes were centrifuged at 10,000 x g for 5 min, and then removed. The supernatants were decanted and immediately centrifuged and all the remaining water was removed from the pellet using micropipette.

The pellet contained the proteins. Copper solution (100 ml) and 400 µl of distilled water were added to each Eppendorf ® tube, vortexed briefly to dissolve the precipitated protein. Working color reagent I ml was added to each Eppendorf ®) tube, mixed on a vortex shaker and incubated at room temperature for 15- 20 min. The absorbance of each sample and standard was read at 480 nm using water for blank as a reference. The absorbance readings were taken within 40 minute after addition of the working color reagent. Standard curve of BSA was drawn on Microsoft Excel ®. It depends on the relationship between standard (BSA) concentrations and absorbance reading in a spectrophotometer. Unknown samples for liver, heart. Lung organs and HT29 cancer cell line of protein concentrations were calculated. The preparation results have shown in Table 4 in theresults.

Dot Blot assay

Materials and apparatus

The materials and chemical for dot blot assay were of analytical grade and includes ELISA well plate 96 wells (Nunclon / Denmark), PVDF membrane 0.45µm pore size (Invitrogen), PBS, 0.3% H2O2 (Systerm), IBMR3 Mabsupernatant, rabbit anti mouse-HRP, serotype IgS (Zymed/ Invitrogen immunodetection), DAB substrate (Invitrogen), bioImaging machine (Chemigenius), protein samples of Balb/c mouse, fromheart, liver, lung, and HT 29 cell line.

Table.1 Spectrophotometer reading of standard BSA sample

Number of Sample	BSA volume µl	Concentration 2µg / µl	O.D. Spectrophotometer reading
1	0	0	0.798
2	5	10	0.777
3	10	20	0.684
4	15	30	0.605
5	20	40	0.525
6	25	50	0.464

Table.2 Method of quantification of protein for three organs and HT29 by spectrophotometery

Sample	
Sample Volume / µl from each sample	15µl
Precipitant / µl	500
Vortex and incubate 2-3min at RT	
Co precipitant	500
Mix briefly by vortex mixer	
Centrifuged at 15000 rpm for 5 min	
Take pellet after decanting supernatant	
Add 400µl H2O + 100µl Copper solution	500
Vortex Briefly to dissolve the precipitated protein	
Added 1ml of working color reagent to each tube mix by vortex	
The sample mixed for few second	
Absorbance of sample and standard at 480 nm was read	

Method

The aims of dot blot assay were to find the best dilution for antigen and anti-body with low and best background. Small circular pieces of polyvinylidene difluoride (PVDF) were placed in each well of an ELISA plate. In Table 3, the plate was divided horizontally in to four parts; each part contains three wells, starting from the left in the upper row. The heart, liver, lung and HT29 samples, three dilution of 2µlwere used on each sample size: 1/5 and 1/25 diluted with (5% skimmed milk powder TBST) and without dilution concentrated protein sample in sequences (con.).

Blocking buffer 50µl (200 µl of normal rabbit serum + 4800 µl of 5% skimmed milk dissolved inTris-Buffered Saline and Tween 20(TBST) was added to each well and incubated for one hour, then blocking buffer was removed without washing. 5µl of primary IBMR3 antibody was added to

each well incubated for one and a half hour and then washed adding to well 50µl washing buffer in a shaker 3x10 minutes.

Different dilutions of secondary anti body (rabbit anti mouse–HRP), (1/25, 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200) was used. Then 5µl of concentration 1/25 was added to the first well line from left until the last well. Then 5µl of concentration 1/50 was added in the second line until the last dilution and so on. This was incubated for 1hr and washed 3x10 with 50µl washing buffer on a shaker. Then 5µl of 3, 3-Diaminobenzidine tetra hydrochloride (DAB) substrate was added until brown color developed. Plates were washed with water on shaker and excess water removed by pipette; image captured with the use of BioImaging system as in results in picture number2. And the best dilution for the secondary antibodies and antigens from different samples were explained in Table 5 in the results.

Table 3: Dot blot assay, plat 96/ well using mouse (liver, heart, lung and HT29 cell line) samples

2nd Ab.dil	Heart			Liver			Lung			HT29			
1/25	Ag dil. 1/5	Ag. dil. 1/25	Ag Con.	Ag dil. 1/5	Ag Con. 1/25	Ag. Con.	Ag Con 1/5	Ag Con 1/25	Ag Con.	Ag Con. 1/5	Ag Con. 1/25	Ag Con.	A
1/50	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	B
1/100	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	C
1/200	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	D
1/400	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	E
1/800	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	F
1/1600	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	G
1/3200	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	1/5	1/25	Con.	H

2µl of each sample was added in each well in different concentration 1/5, 1/25, without dilution
50µl blocking buffer was added to each well and incubated for one hour
Primary IBMR3 supernatant added in each well 5µl and incubated for one and half hour
Washing buffer was added to each well 50µl three times for 10 minutes
Secondary Abs in different concentration were added horizontally for each concentration
Washing buffer was added to each well 50µl three times for 3 minutes
DAB substrate was added to each well 50µl until brown color will be clear then distilled water was used to stop reaction

Results

Result of standard bovine serum albumen curve as in figure 1.

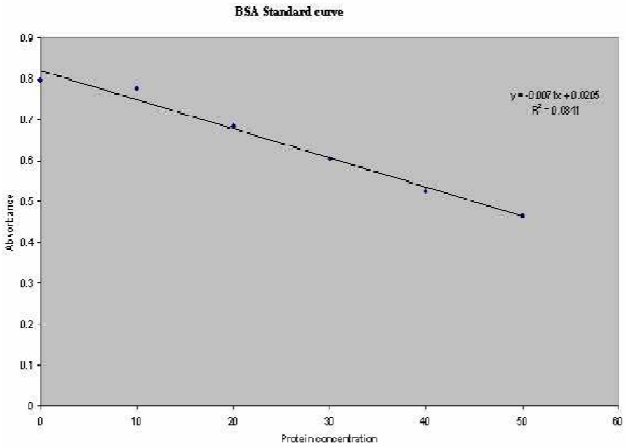

Figure 1, BSA standards curve

The results of liver, lung, heart organs and HT29 cancer cell line of IBMR3 Antigens

The three organs and HT29 cell line of IBMR3 antigens protein were analytical; the concentration were quantified for samples using the same experimental conditions with the same concentrations. The absorbance of samples and optic density were detected using a spectrophotometer. The concentration as in table 4, for the protein of liver(5.093) µg/1 µl, lung (5.018) µg/1 µl, heart (2.075) µg/1 µl, and HT29 (3.328)µg/1 µl.

Table.4 Unknown Balb/c mouse samples spectrophotometer absorbance and protein concentration reading

Rank of conc.	Mouse organs sample	1st.OD reading A.	2nd. OD reading A	Mean reading	Protein concentration µg / 15µl	Protein concentrationµg/1µl
1	**Liver**	**0.288**	0.268	0.278	76.408	5.093
2	**Lung**	**0.341**	0.231	0.286	75.281	5.018
3	**Heart**	**0.647**	0.552	0.5995	31.126	2.075
4	**HT29**	**0.562**	0.370	0.466	46.929	3.328

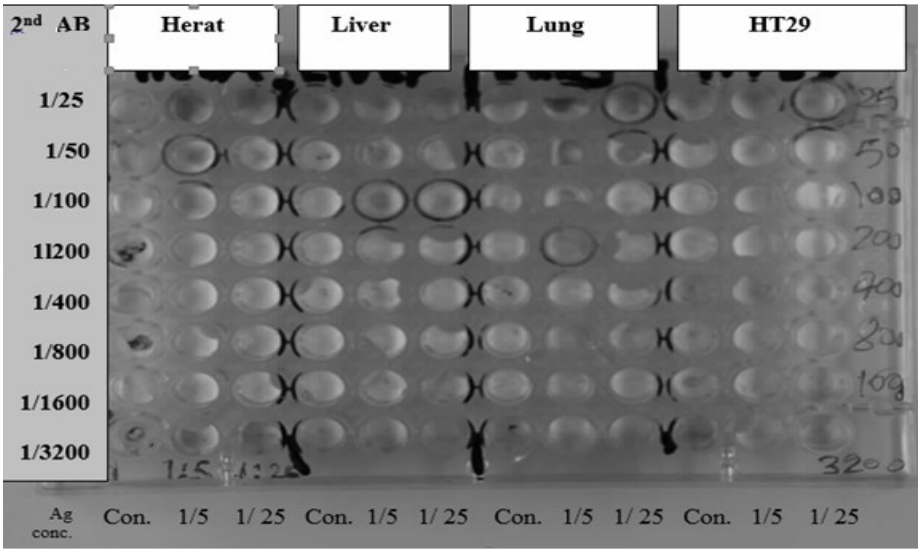

Figure 2: Dot blot assay plat 96/ well / under bio imaging system using 4 protein samples with secondary rabbit anti mouse Igs -HRP.

Note: The best color result in the best low background Organ Sample protein dilution 2nd.

Table 5: The result of best dilutions of four samples with best dilutions of rabbit anti mouse secondary antibody Igs –HRP in dot blot assay

Organ	Sample protein dilution	2nd. Anti body R. anti M.
Heart	1/5	1/50
Liver	1/25	1/100
Lung	1/25	1/25+1/50
HT29	1/25	1/25

Dot blot Results

The figure 2, shows the best color result that gives best low background in mouse samples , the results reading was presented in table 5 , best dilution for the antigen in protein samples for the heart was (1/5), liver 1/25, lung 1/25, HT29 1/ 25.

The best dilutions of secondary antibodies were 1/50 with heart, 1/100 with liver, 1/25 and 1/50 with lung, 1/25 with HT29.

Discussion

The research regards as the first study in this filed to determine the best and lowest dilution for liver, lung, heart Balb/c mouse and HT29 cell line to give clear and good back ground on PVDF membrane, in these results, the primary monoclonal anti bodies IBMR3 may be revealed in connections with similar molecules, and indicated that the recognized antigens may be different domains of the same molecule, or the same liner domain on different types of molecules [1 & 2].

Conclusions

The results from this study suggest that the IBMR3 antigens are expressed in all investigated organs , heart, lung and liver and cancer cell line HT29, but the expression pattern varied from organ to another. This might be highly indicative for expression profiles of this antigen in the same sample or even in the sample of different origins, as recognized by the antibody that gave different reading in the IBMR3 Ag expression. The expression of IBMR3 Ag in cell lines (HT29), this might also be indicative of the fact that IBMR3 antibody can express or recognize the same epitope in different molecules having different relative molecular masses.

Recommendations

MAbs IBMR3 may be useful to identify and study the roles of these molecules in normal tissue and cancer cell lines. This may help in future to use IBMR3 MAbs in cancer tissue and cells to identify IBMR3 Ag to be indicator for some specific type of cancer, that is, it has potential to be a future cancer marker.

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