ANALYSIS OF THE EXPRESSION OF IBMR3 ANTIGEN IN CELLS AND TISSUES FROM RODENTS

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By

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بسم الله الرحمن الرحيم

وقل اعملوا فسيرى الله عملكم ورسوله والمؤمنون وستردون الى عالم الغيب والشهادة فينبئكم بما كنتم تعملون

صدق الله العظيم

سورة التوبة اية ١٠٥

DEDICATION

I would like to dedicate my thesis to the souls of my parents, teachers who taught me in primary and intermediate level. May Almighty Allah gives peace to their souls and bless them with Paradise.

QUTAIBA ALRAWI

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Ag	Antigen
Ab	Antibody
ADH	Anti diuretic hormone
ALL	Acute lymphoblast leukemia
AMI	Antibody-mediated immunity
AML	Acute Mylogenous leukemia
AMI	Antibody-mediated immunity
ACTH	Adrino cortico troffic hormone
APS	Ammonium persulfate
APHIS	Animal and Plant Health Inspection Service
BSA	Bovine serum albumin
CuSO4.5H2O	Copper Sulphate
Cm	Centimeter
C◦	Degree Celsius, Centigrade
C f	Congenital malformations
CSFS	Colony stimulating factors or (interleukins)
CMI	Cellular and Molecular Immunology
CMI	Cell-mediated immunity
DAB	3, 3'-diaminobenzidine tetra hydrochloride
etc	Et cetera
et al.	Et alli, "and others"
ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylene DiamineTetra Acetic Acid
FITC	Fluorescent isothiocyanate
Fc region	Fragment crystallizable region
FCR	Fragment crystallizable receptor
Fab	Fragment antigen binding
g	Gram
HCG	Human chorionic gonada troffin

HIL-4R	Human interleukin -4 receptor
H CGT	Human chorionic gonada troffin
HSCS	Hemapoietic system cells
h	hour
SCF	Stem cell growth factor
Ig	Immunoglobulin
IgE	immunoglobulin E
IL	Interleukin
IHC	Immunohistochemistry
IVIG	intravenous immunoglobulin
KD	Kilo Dalton
Kg	Kilo gram(s)
L	Liter
mab	Monoclonal antibody
M	Molar (mol/L)
Mm	Millimole
ml	Milliliter(s)
MSF	Phenyl methyl sulphonyl fluoride
mg	Milligram(s)
mm	Millimeter
mg /kg	Milligram per kilogram
µg	Microgram
µl	Microlitre
µm	Micrometer
NRS	Normal Rabbit serum
NK	natural killer
nm	Nanometer
O.D.	Optical density
PBMC	peripheral blood mono nuclear cells
PTH	Parathyroid hormone
PVDF	polyvinylidene fluoride

PMSF	Phenyl methyl sulphonyl fluoride
PBS	Phosphate buffer saline
PSA	Prostate specific antigen
R.B.C.	Erythrocytes
R. A. M.	Rabbit antimouse
RF	Retention factor
RPMI 1640	Ross well Park Memorial Institute
Rpm	Rotor per minute
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis
S	second
TBST (Washing buffer)	Tris- HCl buffer Saline+ Tween 20
UV	Ultra violet
USDA	U.S. Department of Agriculture's
APHIS	Animal and Plant Health Inspection Service

Analisis Pengekspresan Antigen IBMR3 pada sel dan tisu dari rodensia

ABSTRAK

Antibodi monoklonal (mab) dan pecahannya telah digunakan secara meluas untuk tujuan diagnostic dan terapeutik. Sel hibridoma IBMR3 telah dihasilkan dari kajian terdahulu. Supernatan sel kultur bagi sel hibridoma telah dikumpul dan disimpan pada suhu -20ºC. Matlamat projek ini adalah untuk mengkaji profil ekspresi antigen IBMR3 di dalam tisu mencit dan tikus, sel-sel HT29 dan 3T3 dengan menggunakan kaedah immunoblotting dan perwarnaan immunohistokimia menggunakan kaedah peroksida. Di dalam kajian ini pengekspresan antigen IBMR3 telah dinilai di dalam 80 organ yang berbeza dari mencit Balb/c dan tikus Sprague Dawley. Sel-sel yang telah dikenalpasti, sel kanser kolorektal manusia HT29 dan fibroblast 3T3. Tisu ini kemudian di laisiskan dengan penimbal laisis dan kepekatan laisis yang sama turut dijalankan pada 12% SDS-PAGE. Jalur protin yang terpisah, dipindahkan ke membran PVDF/Polivinildin diflorida untuk immunobloting. Hasil immunoblot telah dibuat analisis densitometrik untuk menentukan berat molekul, puncak ketinggian dan isipadu mentah untuk setiap protin. IBMR3Ag telah menunjukkan ekspresi di dalam tisu-tisu mencit dengan berat molekul yang paling tinggi (98.22 kDa) dan bilangan jalur protin yang paling banyak dikesan di dalam otot dan paling rendah di dalam jantung (75 kDa) dan hati (78.68 kDa). Di dalam tikus, lebih banyak jalur protin dikesan di dalam otak dan ginjal manakala hanya 4 jalur protin dikesan di dalam limpa. Berat molekul yang paling tinggi dikenalpasti di dalam otak (120.83 kDa) ginjal (85.48 kDa) dan limpa (73.42 kDa). Ekpresi antigen IBMR3 paling tinggi didapati di dalam paru-paru dan ekspresi paling rendah di dalam otak untuk kedua-dua mencit dan tikus. Kaedah pewarnaan

immonohistokimia dengan teknik peroksida telah digunakan untuk mengkaji profil ekspresi antigen IBMR3 di dalam cebisan tisu-tisu beku yang berbeza bagi mencit dan tikus. IBMR3 Ag yang positif boleh diperhatikan di bawah mikroskop bercahaya sebagai perang cair dan berubah berdasarkan kepekatannya samada lemah, sederhana dan legap. Ekspresi IMBR3 Ag membantu menganalisa profil bagi berat molekul, puncak ketinggian dan isipadu mentah. Keputusan ekspresi positif berbeza di antara kaedah immunobloting dan pewarnaan immunohistokimia untuk IBMR3 Ag di dalam bahagian tisu yang berbeza, membantu menentukan tahap antigen IBMR3 terhadap parameter patologi. Keputusan kajian ini menjelaskan bahawa antigen IBMR3 telah memberikan ekspresi yang berbeza di dalam tisu mencit, tikus dan sel. Kajian seterusnya akan dicadangkan pada masa hadapan untuk mengenalpasti dan mengkaji keadaan tindakan semulajadi antigen IBMR3 dan potensinya di dalam perjalanan pembangunan tisu.

ANALYSIS OF THE EXPRESSION OF IBMR3 ANTIGEN IN CELLS AND TISSUES FROMRODENTS

ABSTRACT

Monoclonal antibodies (Mab) and their fragments have been widely used for diagnostic and therapeutic purposes. IBMR3 hybridoma cells were produced in a previous study. The cell culture supernatant of the hybridoma cells was collected and stored at -20 °C freezer. The aim of this research is to investigate the expression profile of the IBMR3 antigens in different tissue of mouse, rat and cell lines HT29, 3T3 by using immunoblotting technique and immunohistochemical staining using the peroxidase technique. In this study the expression of IBMR3 antigen was evaluated in eighteen different organs taken from Balb/c mouse and Sprague Dawley rat, established cell lines, human colorectal HT29 cancer cell line and mouse fibroblast 3T3. Various mouse and rat tissues were snap- frozen and stored in liquid nitrogen. For immunoblotting six micron frozen sections of the various tissues were prepared and collected in Eppendorf tubes. The sections were then lysed in lysis buffer, and equal concentrations of lysates were run on 12% SDS-PAGE. The separated protein bands were transferred to PVDF/ polyvinylidene difluoride membrane for immunoblotting. Immunoblotting were subsequently subjected to densitometric analysis to get the value of molecular weight, peak height and raw volume of the protein band (Burnette, 1981). IBMR3Ag was expressed in mouse tissues with the highest MW (98.22) kDa and highest number (nine) of bands detected in muscle and the lowest MW in heart (75.00) kDa and liver (78.68) kDa with lowest number (2) of bands. In rat, more bands (eight) were detected in brain and kidney. While in spleen only four protein bands were detected. The highest MW was also indicated in brain (120.83 kDa), kidney (85.48 kDa)

and 73.42 kDa in spleen. In both mouse and rat, highest expression of IBMR3 antigen was found in the lung and lowest in the brain. The immunohistochemical staining method was used in peroxidase technique for the investigation of the expression profile of the IBMR3 antigens in different frozen tissues from mouse and rat. The positive IBMR3 antigen were seen under light microscope as brown color, the results between weak, moderate and intense. Expressed IBMR3 antigens help to analyze the profile for molecular weight, peak height and raw volume. The different results in immunoblotting and immunohistochemistry staining gave positive expression for IBMR3 Ag in different part of the tissue sections, this shed more light on, whether the level of IBMR3 antigens are associated with pathological parameters. The results from this study suggest that the IBMR3 antigens were differentially expressed in mouse, rat tissue and cell lines. It was recommended that further research be conducted to identify and investigate the nature of the IBMR3 antigen and its potential role in tissue development.

CHAPTER 1

INTRODUCTION

1.1 Monoclonal antibody

The uses of monoclonal antibodies (Mabs) have been accepted for diagnosis and therapeutic medical indications, especially in oncology (Van Dongen *et al*., 2007: Abouzied *et al*., 1993; Cheung *et al*., 2002; Emanuel *et al*., 2000). Monoclonal antibodies can be used for the diagnosis of a specific antigen protein in a cancer cell line, normal cell line, normal or cancerous organs, bacteria, virus, parasite, food and blood. There are several types of methods for such studies like immunohistochemistry, immunoblotting and blot assay.

The expression can be seen by the use of a labeled antibody with radioactive element, colloidal gold, fluorescent dye or enzyme. Therapies based on treatment with antibodies are being extensively investigated in several clinical trials during the past few years. Monoclonal antibodies can be generated against most target antigens, purified and split into fragments. Monoclonal antibody (Mabs) has the ability to conjugate with radio nuclides, toxins, enzymes or drugs.

Such methods are used in laboratories for common techniques in many medical research and diagnostics (Hawkins *et al*., 1992). Mabs are preferred to be used in western blot, ELISA, immunohistochemistry, cell sorting (cell isolation) by (FACS) Fluorescent-activated cell sorting, cell depletion, confocal microscopy, protein purification and Imaging because of lower background interference**.**

By nature, Mabs originate from one specific clone with higher specificity, purity, consistency and attractive for one epitope of the antigen. However, these antibodies require secondary antibodies which are “prepared to measure” both single

and simultaneous measurement of multiple analyses (Spinks, 2000). The expression of IBMR3 in different organs and in cancer cell line encouraged to be employed as a future strong marker in diagnosis of cancerous diseases.

1.2 IBMR3

1.2.1 Introduction of IBMR3 Ag

Monoclonal antibody (Mab) is a single type of antibody. Cell line fusion between stimulated B-cell with myeloma cell which produce hybridoma cell is shown in Figure 1-1. The monoclonal antibody then will produce by cloning of a single hybridoma or single parent cell line; sometimes naturally, myeloma cells produce single Mab (Hawkins *et al*., 1992).

Figure 1.1: False color scanning electron micrograph of hybridoma cells used to produce monoclonal antibodies for treating cancer (Hawkins, *et al*., 1992)

IBMR3 is a monoclonal antibody, IgM isotype, produced by Ishak Mat in mice (Hara and Mat, 2004). Many antibodies have been raised using synthetic peptides corresponding to selected amino acid sequence for protein encoded by various genes (Walter and Doolittle., 1983). One of the attractive results of using this approach is that antibodies raised may cross react with native proteins (Mat, 1992). In another study generated mouse Mab IBMR3, of IgM isotype, using synthetic peptides corresponding to selected amino acid sequences of the (HIL-4R) human interleukin 4 receptor molecules, (Galizzi *et al.*, 1990). Because of mammalian IL-4 receptor proteins.

Previously it was shown that Mab IBMR3 does not block IL-4 (Mat, 1992). On the other hand Mab IBMR3 showed recognition and binding with relative molecular mass of 100 KD and 50 KD with minor affiliation at 200KD (Mat,1992). Mab IBMR3 was used to analyze the expression of IBMR3Ag in normal (PBMC) peripheral blood mononuclear cells, established cell lines, AML (acute mylogenous leukemia) and ALL (acute lymphoblastic leukemia), after analysis by flow cytometry and immunoblotting techniques. The results showed a weak response in resting PBMCs on surface staining with high cytoplasmic levels of the antigens. Similar expression in ALL and AML were observed except that of the AML two samples since they showed surface expression while all of the ALL-B and ALL-T specimens were non- reactive (Hara and Mat, 2004).

1.3 Expression patterns / significance of IBMR3 Ag

Flowcytometry results suggest that the IBMR3 antigens are mostly indicated in the cytoplasm rather than on the cell surface. IBMR3 Ag has shown different expression in different cells. However, surface expression may occur in certain type of cells as seen in two cases of AML (Acute Mylogenous leukemia). Immunoblotting analysis suggests that IBMR3 Ag expression on the cell surface is inducible.

They showed that although all cells expressed the IBMR3 Ag, some cells differed in their expression. For example HepG2 did not express the 230kDa molecule but 45kDa molecules can express.

The nature of the Ag recognized by Mab IBMR3 has not been determined and the significance of this observation is not known. Nevertheless, Mab IBMR3 might recognize the same epitope which is shared by molecules having different molecular masses (Hara and Mat, 2004). Mab IBMR3 may be useful to identify and study the roles of these molecules in normal and transformed cells. These results suggest the possible association of up regulated IBMR3 Ag expression in malignant cells and cells with poorly differentiated morphology. From these studies one can speculate that IBMR3 Ag might have a role in tumor development and that its expression pattern may have a significant value in cancer prognosis (Hara and Mat, 2004).

1.4 Antibody

1.4.1 Characteristics of a good antibody

The main requirement for a good antibody is that it should have high affinity for its antigen. Generally, antibodies are classified into three groups; IgM, IgG and IgA. Their concentration, specificity and isotype can affect or rather determine the action of an Ab. (Casadevall and Pirofski, 2003). Antibodies connect with specific antigens at

suitable sites and not with other unrelated antigens. The attachment site, commonly known as fitting site, establishes the strength of connection between antibody and antigen (Roitt *et al.,* 2006). Antibody applied in (IHC) immunohistochemistry as much as immunocytochemistry and it is applied in histopathology and cell biology (Gatter *et al.*, 1985). The fragments of antibodies can interact with the amino acids on the cell which form antigen (molecules) or binding site of the antibody.

1.5 New concepts in antibody-mediated immunity

The immuno regulatory properties of antibody have been known since the beginning of passive immunization experiments. The development of the field of immunology started in the beginning of 20^{th} century.

The functional aspects of antibody-mediated immunity (AMI) stagnated in the 1960s. The potential to adjust an immune response to immunize antibody with binding site has been established more than ten years ago (Antoniou and Watts, 2002; Rafiq *et al*., 2002; Rhodin *et al*., 2004). After knowing the function of antibodies, the available Abs preparations were limited to polyclonal immune sera. The discovery of T cells helped to improve T cell-mediated immunity.

At the end of 1980s the development of monoclonal antibody technology started, followed with discovery of Fc receptors (FcR) with the generation in mice.

All these helped to get more information which made possible interesting studies in the basic mechanisms of AMI antibody-mediated immunity (Reichert and Pavlou, 2004).

Antigen -specific immunity, like synthetic molecules, helped in activation of innate immunity system by stimulating (TLRs) T lymphocyte receptors, B cells, macrophages and dendritic cells. This activation, mediated after increasing Ag-specific

Abs (IgG and IgM), helps to trigger strong inflammation that in turn helps to protect body from bacterial, viral and parasitic infections (Pedras-Vasconcelos *et al*., 2006).

Polyclonal and monoclonal reagents can be used either alone or mixed with antigens to increase or protect immune responses, malignant tumors and infectious agents as well as to decrease harmful responses related with autoimmunity, inflammation and hypersensitivity (Igietseme *et al*.,2004; Nicodemus *et al*.,2002; Stager *et a*l., 2003). The stable activated B cells perform important role in an independent homeostasis by generating IgM secreting cells (Agenes and Freitas, 1999). Use of passive Abs also increases the immunological protection against biological weapons. Passive antibodies can provide immediate immunity by stimulating antibody-mediated immunity (AMI) against a biological agent (Buchwald and Pirofski, 2003; Casadevall, 2002). Continuous improvement of studies in technological fields of antibody-mediated immunity (AMI) and the demand of urgent therapies for new and emerging diseases helped the discovery of new Ab functions that have wide classical views of (AMI) antibody-mediated immunity. The B cell is the origin of the whole Ab molecule. In addition, the study of (AMI) antibody-mediated immunity has also come from studies with Ab fragments and antibody-derived peptides.

T cells are the principal effector cells of specific cellular immunity and they have the ability to differentiate functionally to $CD4^+$ cells which serve as helper cells and $CD8^+$ cells which serve as cytotoxic cells (Sharma, 1999).

The antibodies can act as positive and negative regulators of inflammation and (CMI**)** cell-mediated immunity. They have the ability to amplify or suppress the inflammatory response. Abs has anti-inflammatory effects which can be observed by intravenous injection of immunoglobulins for the treatment of inflammatory conditions (Bayry *et al.* 2003).

Immunoglobulin M (IgM) molecule has powerful activators of the complement system which is pro-inflammatory. IgM has the property of early presence in the course of infection and defense against certain experimental bacterial and viral infections by amplifying the immune response (Casadevall and Pirofski, 2003).

1.6 Classical views of antibody-mediated immunity (AMI)

Antibody molecules have two domains, the antigen binding region which contains variable (V) region elements and a constant (C) region. The V region has binding ability to antigens with several forces such as hydrophobic, ionic, hydrogen bonds and Van der Waals interactions (Casadevall and Pirofski, 2004).

The C region includes a fragment crystallizable (Fc) segment which determines the antibody's serotype and functional characteristics, such as its half-life in serum, ability to interact with FcR and complement activation. There is high capability of binding between Fc region and cellular receptors with some humoral components of the immune system such as the complement system. Ab functions as a junction bridge between a microbial antigen and the immune system (Casadevall and Pirofski, 2004). The function of specific Ab takes place in two types of actions, direct and indirect action:

1- Direct or classical, such as virus neutralization and toxin, regarded as antimicrobial effect. Ab has ability to directly interact as bacteriostatic for example *Escherichia coli* lipopolysaccharide. In addition it has ability to interact directly with release of iron chelator, enterochelin. Antibodies are commonly used in therapeutics in eradicating bacteria (Fitzgerald and Rogers, 1980: Kozel *et al.*, 2004). IgM and IgG antibodies have direct damaging action on the surface proteins of *Borrelia burgdorferi* which is very essential to protect microorganism from outer affections, leading to a bactericidal effect

in the absence of complement (Connolly and Benach, 2001; Connolly *et al.*, 2004). Also, Ab binding can help to remove parasites in certain gut disorders and luminal parasites (Carlisle *et al.,* 1991).

2- Indirect activities need at least one more element of the immune system to interact with Ab such as opsonization and complement activation.

After development of a connection between Ab-mediated complement and Ab, activation results in opsonophagocytosis function for some microbes. Antigen can be eaten by a phagocytic cell after linking with an antibody, known as opsonin.

The best antibody in opsonin is IgG. The IgG antibody binding with Fc receptors on other types of cells, results in the activation of other functions (Kozel *et al.* 2004).

Another important mechanism is the activation of natural killer lymphocyte cells (NK), which act as cytotoxic and is antibody mediated against tumors and microbes. Passively, antibodies when transferred inside the body can neutralize and blocks inter virus infection (Haigwood *et al*., 2004**:** Kunkl *et al.,* 1992). Antibody-mediated response has ability and power to restrict and define regions of each immunogen (Rhodin *et al.* 2004). Systemic immunization consists of antigen coupling with Mab. This increases the number of Mab producing hybridomas against an antigen (Brady *et al*., 2000).

1.7 Structure and production of immunoglobulin

The antibodies are divided into five classes: IgD, IgA, IgM, IgE and IgG. Each antibody consists of a basic monomeric unit and four chains. These chains consist of two heavy and two light chains: Kappa (κ) light chains or Lambda (λ) light chains. The whole antibody consists of a (constant and variable) region.

The fragment antigen binding (Fab) region of variable contains NH_2 terminal end and the fragment crystallizable (Fc) constant contains COOH terminal end

(Schwartz, 1995). Each antibody variable end contains only one kind of antigen binding site. The fragment crystallizable (Fc) portion has the ability to identify class and binds with some cells which have FcR terminal.

The FC receptors usually bind to plasma proteins cells (Kuppers *et al*., 1999). B cells have receptors on a special surface that best fit antigens involved in helping the multiplication (affinity maturation), providing a means whereby more specific antibodies with greater similarity can be produced (Schwartz, 1995).

The most commonly found class is IgG (Myara *et al*., 1991). Any immunogen which induces an immune system response will result in the production of IgM and IgD iso-types which are situated on the B-cell surface as recognition receptors. Many of the immature cells produce low-affinity antibodies that bind to multiple antigens (Diamandis, E.P., 1996; Bouvet and Dighiero, 1998).

Autoimmune reaction initiates the production of antibodies in normal sera in large quantity and shows alterations in specificity in different pathological situations when the immune system gets exposed.

IgM, IgG, and IgA, natural antibodies contribute to the establishment and maintenance of immune memory especially when the immune system is exposed to pathogenic or physiological action like trauma or injury (Leslie and Martin, 1978; Rostenberg and Penaloza, 1978).

IgD antibody has the ability to regulate the protective response of IgG, IgM, and IgA isotypes which otherwise interfere with viral replication (Moskophidis *et al*., 1997).

Immunoglobulin E (IgE) has the ability to attach with plasma cell membrane of mast cell surfaces because they have many IgE receptors (FceR). High-affinity receptor for IgE (FcERI) is easily expressed on mast cells and basophils, which help cell

activation and immediate release of allergic mediators during parasitic infections (Molfetta *et al*., 2005; Metcalfe *et al.*, 1997).

Activation of human mast cells through the high-affinity receptor for IgE (FceRI) underlies atopic allergic reactions (Okayama, 2000). These IgE molecules act specifically for helminthes which encourages mast cell binding to the parasite.

IgG immunoglobulins play a role of opsonin in phagocytosis, of *Cryptococcus neoformans*, by macrophages which help phagocytic cells to eat (opsonate) antigen (Kozel and Mc Gaw, 1979).

Immunoglobulin E (IgE) is best known for such opsonitic activity because they mediate binding of mast cells to parasitic helminthes (Lukacs *et al*., 1996). IgE specific to *Helicobacter pylori* and *Staphylococcus aureus* has been reported in patients with peptic ulcers and topic dermatitis. However, IgG is not typically generated against other bacteria inspite of FceR availability (Aceti *et al.*, 1991; Leung *et al.*, 1993).

The class of each immunoglobulin is different from other serum proteins and depends on factors like, solubility in aqueous solution, isoelectric point, electrostatic density and the molecular size. These differences in characteristics help to isolate the immunoglobulin and fractionate it to different classes (Hudson and Hay, 1980).

1.8 Immunogenicity for the production and standardization of antibodies

When an immune system has been exposed with an infection, natural antibodies created on, or exposure of the immune system with vaccination, immunogen helps to create a new antibody which can be used in diagnostics or for research and therapeutic purposes, the immunogen have the ability to raise polyclonal antibodies and fragments of monoclonal antibody with hybridoma cell lines (Abouzied *et al*., 1993; Skerritt *et al.*, 1996; Cheung *et al*., 2002; Korban *et al*., 2002; Darko *et al.*, 2005).

The quality of antibodies produced by immunization depends on the nature of the selected host, the quantity of the antigen administered per dose, the frequency of exposures of antigen and the time intervals between exposures (Emanuel *et al*., 2000; Doria-Rose *et al.*, 2005).

Individual antigens have been used in immunization as immune complexes (IC) improved the enhancing of the immunogenic of soluble molecules and increase the number of monoclonal antibody (Mab) producing hybridomas against an antigen, and to elicit antibodies specific for poorly immunogenic epitopes (Brady *et al.*, 2000).

Polyclonal anti-sera are a term used when the serum contains a collection of antibodies or when immunoglobulins are isolated from such sera (Leenars *et al*., 1999; Cachot *et al*., 1998: Akerstrom *et al*., 1985).

Polyclonal antibodies can be used in research and used in, immunoflourescence, immunohistochemistry and detection of antigens by ELISA, Western blots, immunoprecipitation procedures and immuno electron microscopy (Schipper *et al.*, 1991; Werner *et al.*, 1996; You *et al*., 2002; Sasano *et al*., 2003).

Synthetic epitopes can be used to elicit anti-native protein antibodies, synthetic peptides have been used for producing Mabs and polyclonal anti bodies with the same or better affinity for proteins which generally observed for anti protein responses (Bahraoui *et a*l., 1986**;** Laman *et al*., 1992).

The protein site of the antigen has the ability to connect with large molecules, like antibodies. The attraction between the antibody and antigen has been improved when the hydrophilic protein part is exposed (Westhof *et al*., 1984; Karplus and Schulz., 1985; Fieser *et al*., 1987).

1.9 Cancer

1.9.1 Tumor immunology

Tumor immunology deals with the study of immune response towards tumor and studies properties of the antigens of the tumor cells. In addition it includes a study of the immune response towards cancer cells, the immunogenic effects to the host and the changes of the immune system to identify and eradicate tumors. Any tumor to initiate an immunological response must have characteristic antigens, any immunological approach to the treatment of cancer should be specific to destroy tumor cells only, while normal tissue should be unaffected (Castro, 1978).

Cancer immunology deals with the study of major advances in molecular and cellular immunology and determines the overall understanding of the complex and high rate of interactions between the immune system and tumor cells. The tumor–immune system interactions may act in a strong immune antitumor response or tolerance to tumor-associated antigens (Delgado, 2002).

Tumor cells have been shown to be carrying antigens on their surface which can be detected by the immune system in the tumor host. The immune system has the ability to recognize any changes in cell surface and responds to an abnormal change or to the formation of tumor cells.

Some cases lead to the escape of transformed cells from the immune system and it has been observed that it leads to the formation of malignant tumor. This lead to the opinion that the immune system is not effective enough to eradicate cancer. Advanced studies have demonstrated that the immune system cells can be increased in number to raise the immune sensitivity against the tumor disease and becomes a potential means of treatment (Ritz and Schlossman, 1982; Abbas and Lichtman, 2005).

1.9.2 The causes of cancer

The cause of cancer may depend on the type of the cancer on the abnormal changes in gene regulations (Hall *et al*., 1998). The cause of cancer can be divided in several cases, like physical, chemical, environmental immune deficiencies, diet, drugs and gene mutation.

There are several DNA sequence mutations as strong evidences, which cause cancer in human and mice (Watson *et al*., 2003). Chemical carcinogen causes mutations in proto-oncogene or tumor suppressor gene which helps abnormal growth of the mutated cells to transform and develop cancer cells (Weisburger and Williams, 1981). Some drugs also contain chemical agents that can lead to cancer death (Daly, 1993). Immune deficiencies also increase probability of malignancies (Bishop, 1999).

1.9.3 Neoplasm

Neoplasm is the term of 'tumor' translated accurately as swelling or a new abnormal growth in any part of the body which has some of similarity to its tissue of origin. Mass of abnormal tissue grows in a persistent fashion and its proliferation does not stop even after the removal of the causative agent (Paul, 1999).

There are two types of neoplasms: malignant & benign. The common term for malignant tumor is cancer. Classification of human cancer depends on the molecular profile histological appearance which helps in diagnosis and treatment (Morris *et al.,* 1998; Mazzanti *et al.*, 2004).

1.9.3.1 Malignant tumor

Malignant tumor or malignancy means uncontrollable anaplastic or dysplastic cells growth. This growth and invasion is usually carried out via direct lymphatic or haematogenic vessels (Cotran *et al*., 1999). Malignant tumor aggressively invades neighboring and/or adjacent tissues by diffusing in it and ultimately destroying it (Morris, *et al.,* 1998).

Tumor invades lymphatic tissues, nerves, vascular system and distant organ which is referred to as metastases. Cancer cells produce protease enzyme that affects membrane modification to escape through the adjacent cells (Morris, *et al.,* 1998).

Other evidence has shown that cancer is a heterogeneous disease (King, 2000). The formation of the cancer can be divided in two phases, premalignant and the staging process (Cotran *et al*., 1989).

Mutation changes on the cancer cells include growth regulation gene, growth and morphology, antigen expression on the surface of the cancer cell, change in the enzyme level and hormones (Solomon *et al*., 1991**;** Fearon and Vogelstein, 1990). The exposure of radiation through ultraviolet sun light causes skin cancer and leads to inhibition of priming immune system during vaccination (Byrne *et al*., 2006).

1.9.3.2 Benign tumor

Benign tissue tumor is often encapsulated by variably thick fibrous tissue with a smooth external surface. Benign tumor has a slow growth and cells tend to be small, although some times are large (Morris et al., 1998; Mazzanti *et al.*, 2004).

1.9.4 Epidemiology of cancer

Epidemiology is the field of science that deals with the study, contribution of the distribution and determinants of a disease in human population in the world. Cancer expression varies in different malignant tumors that may be distinct mainly by their tumor site in the body e.g., colorectal, breast cancer, brain cancer and etc. or it some times depends on its histological type e.g., Kaposi's sarcoma (McCredie and Coates, 1998).

Since immune system plays a role in tumor check it is thought that immune deficiency is one of the probable aggravating factors of malignancies. For example in AIDS patients, Kaposi's sarcoma, central nervous system lymphoma and non Hodgkin's lymphoma are recognized as AIDS- defining conditions (Bishop, 1999).

1.9. 5 Cancer markers / immunity

Until now there is no single specific marker for tumor diseases. However, in the clinical management it is useful to use a number of tumor markers to aid diagnosis of malignant patients. Some of these markers are produced serologically from different hormones.

Others are produced by glands or by tissues from or turmeric organs. Bronchogenic carcinomas produce marker hormones such as (ACTH) adrinocortico trophic hormone, (PTH) parathyroid hormone, calcitonin and antidiuretic hormone.

Breast carcinomas produce prostaglandins which can be used as a marker. Some of these markers can be used clinically such as human chorionic gonadotrophin (HCG) and prostate specific antigen (PSA) which is mainly produced by choriocarcinoma and prostatic carcinoma. Gastrointestinal tumors also produce some type of proteins, which are only useful for predicting, prognosis and monitoring therapy. Pancreatic carcinomas

produce CA19-9 and DUPAN-2 while high serum levels of CA-125 are used to diagnose ovarian carcinomas. In the clinical settings, presence of urinary para proteins is suggestive of plasma cell myeloma ((Morris *et al.,* 1998).

1.10 Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical instrument, electrophoresis is simple to use, rapid and highly sensitive (Rybicki and Purves, 1996). SDS-PAGE contains sodium dodecyl sulfate which acts as an anionic detergent which help to separate proteins in the cell fractions though polyacrylamide gel electrophoresis. The separation of protein is dependent on the molecular weight of their polypeptide chains (Shapiro *et al*., 1967).

Separation of native proteins on polyacrylamide depends very strongly on the size of the molecules in addition to the charge (Ornstein, 1964). Sodium dodecyl sulfate (SDS), an anionic surfactant that is unusually used to act as hydrophobic binding environments of cell membranes, in addition help in denaturing process of some native state proteins (Xu and Keiderling, 2004). Several protein molecules have been shown to have binding with the dodecyl sulfate ions (Natarajan *et al.,* 1999).

Electrophoresis has become an essential technique for detecting and determining the molecular mass of some of the unknown proteins and characterization of proteins by western blot or mass spectrometry analysis (Sheen and Ali-Khan, 2005**;** Ikeda *et al*., 1998).

These characteristics of polyacrylamide gel are used to separate most proteins and small oligo nucleotides that require a small gel pore size for retardation and separation of proteins and nucleic acids **(**Rybicki and Purves, 1996: Rybicki, E.P.2005). Proteins are amphoteric compounds. Their net charge is dependent on the pH of the medium in which they are suspended.

A protein has a net negative charge when the pH solution has a higher isoelectric point and positively charged when the pH of the solution is below its isoelectric point. The protein net charge depends on the size of protein molecules which is different from protein to protein and under non-denaturing conditions the electrophoresis separation of proteins is limited by both size and charge of the molecules (Rybicki and Purves, 1996).

1.11 Western blot

Electroblotting has been a feature of a large number of laboratories and there are a large number of different apparatus around that can efficiently transfer proteins (or other macromolecules) from polyacrylamide gels to nitrocellulose sheets. Most of these, however, are based on the design of Towbin (Towbin *et al*., 1979). That is, they have vertical carbon / stainless steel / platinum electrodes in a large tank (Rybicki and Purves, 1996). Western blot is used in wide range and is very important in biologically orientated fields, because of ease of use, accurateness and ease of observation.

Western blotting depends on combination of gel electrophoresis with the specificity of antibody detection. Sensitive direct immunoblotting assays are easy and can be rapidly implied methods for detection of antibody specificity using strips of nitrocellulose or polyvinylidene fluoride (PVDF) membranes (Radl *et al*., 1988**;** Boersma *et al*., 1989; Burry, 2000).

Immunocytochemistry has also been widely used for localization of proteins in cells and tissue with antibodies. The connection between antibody and cell proteins depends on two independent criteria which are antibody specificity and type of method used. Parallel strip of electrophoresed protein extract on acrylamide gel can then be stained to expose the protein bands and matched up against the immunostain paper strip (Polak and Noorden, 1983).

1.12 Immunochemistry staining

Immunochemistry staining includes immunocytochemistry and immunohistochemistry, the primary antibody mainly reacts to a specific antigen, combining sites which are known as fragment antigen binding (Fab) in cells or tissue of the antigen protein and not to other proteins (Itoh *et al.*, 1977).

It is a technique in which an antibody reaction is raised against a specific tumor antigen on a histological tissue section and cells. This technique is widely used in tumor identification, diagnosis and classification. Antibodies labeled with fluorescent dyes conjugated with specific reagents such as horse reddish peroxidase (HRP) are used *in situ* to identify an antigen in tissue section (Polak and Noorden, 1983).

Histochemical and cytochemical techniques using immunoperoxidase and immunofluorescence staining technique which is use in histo and cytochemical in some cases provided additional criteria (Bosman and Kruseman, 1979). It is a useful and sensitive assay in detecting specific antigens, gene expression or gene amplification in tumors and normal tissues (Dickson and Lippman, 1995; Taylor, 1980).

1.13 Fundamentals of blood cell biology

Immunology is the science to study the body immunity and explanation of the antibody behavior against any infection or any foreign substances that have entered any living body. The response of immune system includes several type of cells, an antigen when enter the body will stimulate the immune system and encounters special cells called antigen-presenting cells (APCs) the duty of these cells are to capture the foreign antigens.

The defense response, which occurs by such cells, is known as cell-mediated immunity. The remaining soluble macromolecules and proteins in the blood circulation as well as in extra-cellular fluid generate humororal response, thus known as constituents of humoral immunity (Parslow *et al.*, 2001).

Generally, antibodies are classified into three groups; IgM, IgG and IgA. Their concentration, specificity and isotype can effect or rather determine the action of an Ab. (Casadevall and Pirofski, 2003). Antibodies connect with specific antigens at suitable sites and not with other unrelated antigens. The attachment site, commonly known as fitting site, establishes the strength of connection between antibody and antigen (Roitt *et al.,* 2006).

Antibody applies in (IHC) immunohistochemistry as much as immunocytochemistry and it is applied in histopathology and cell biology. The fragments of antibodies can interact with the amino acids on the cell which form antigen (molecules) or binding site of the antibody (Gatter *et al.*, 1985).

The function of immune system is to destroy any foreign substances in any body by direct killing with cytotoxic T cells or by the antibodies. Antibodies have ability for toxify, neutralize directly the venom, insects or snakes toxins bind these antigenic proteins or indirect by promotes the phagocytosis of these toxin by macrophages and

other phagocytes, furthermore antibodies have ability to neutralize the virus by blocking the protein receptors on the virus surface to prevent virus from connection with other cells. Antibodies can help the macrophages and phagocytes in opsonization and phagocyte the target by attachment with Fc receptors on the surface of these cells, after the Ab was coated the foreign body (Roitt *et al*., 2006).

IBMR3 is a monoclonal antibody of IgM isotype, produced in mice (Hara and Mat, 2004). All cell types in the immune system originate from the bone marrow. There are two main lineages that derive from the hemapoietic stem cell: (Hawkins *et al*., 1992).

1- The lymphoid lineage

The lymphoid lineage consists of T lymphocyte (T cells), B lymphocyte (B cells) and natural killer cells (NK cells). The B cell has a membrane-bound immunoglobulin as a receptor by which it has the ability binding with antigens, and every Ig on an individual B cell has one specificity (Hunt, 2007).

2. The myeloid lineage

The myeloid lineage consists of monocytes, macrophages Langerhans cells, dendritic cells and megakaryocytes and the type of granulocytes which includes (eosinophils, neutrophils and basophils). Hematopoiesis can be seen summarized in the schematic over view as below in figure 1.2.

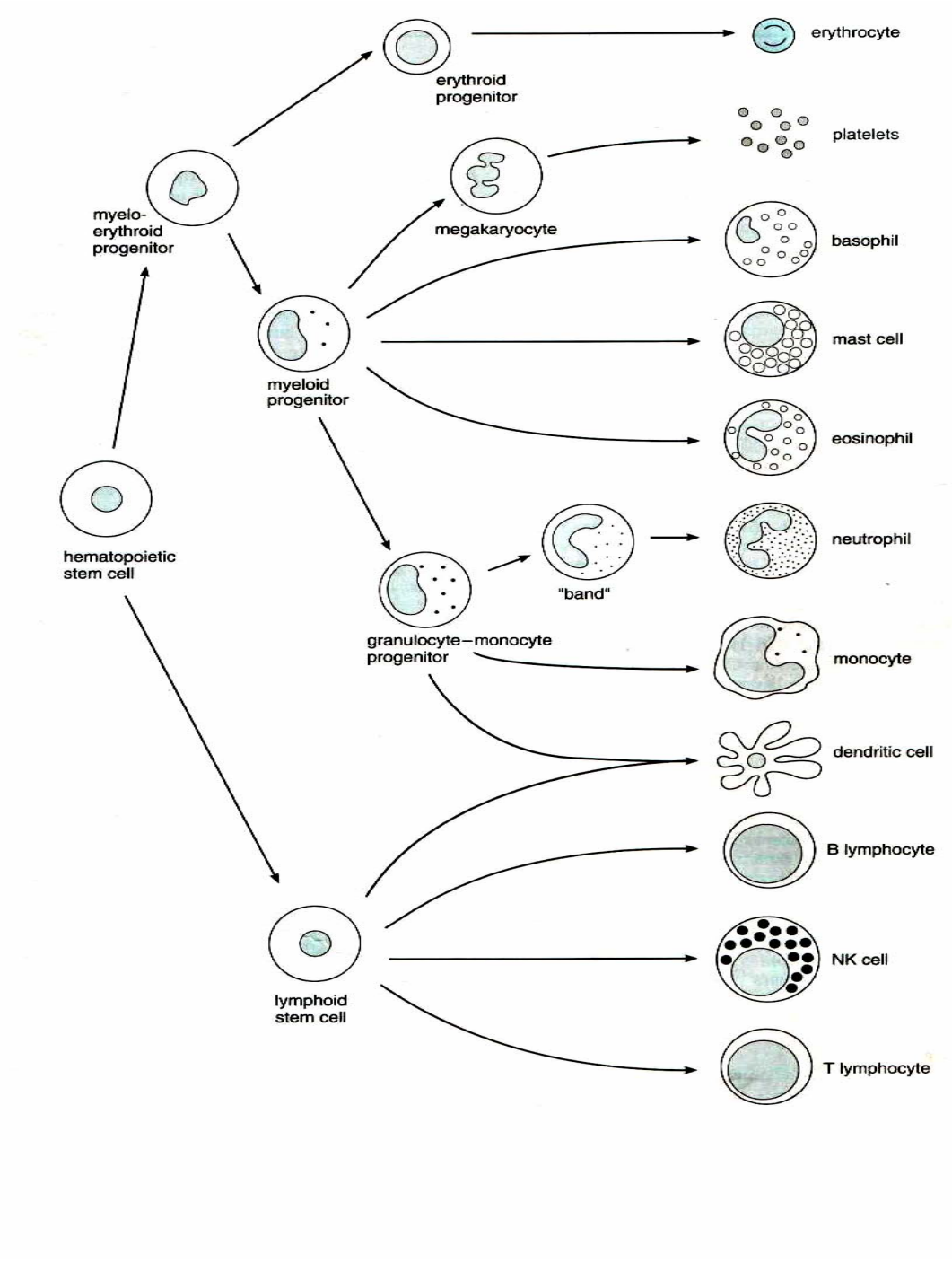

Figure 1.2: Schematic overview of hematopoiesis, emphasizing the erythroid and lymphoid pathways

[Adapted from; Parslow *et al*. 2001]

1.14 Objectives of the study

To determine and investigate the expression profile of the IBMR3 antigens in different tissues and organs of Balb/c mouse, rat and cancer cell line HT29 in addition to mouse fibroblast 3T3 using immunoblotting technique through expression profiling by molecular weight (MW), peak height and raw volume.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media preparations

2.1.1 RPMI 1640 medium

The RPMI-1640 medium (Rosswell Park Memorial Institute) mix (Hiclone, USA) was developed by Moore *et al*., (1967). RPMI 1640 powder contained 2.05mM L-glutamine without sodium bicarbonate (A Perstorp Life Sciences Company). It was dissolved in deionized water to make a final volume of 1 L (Ultra PUM 1.8.2 MΩ-CM).

The medium was first dissolved in 800 ml of deionized water. 2.0 g/ L sodium bicarbonate was added to this solution. The medium was then passed through a filter paper to exclude undissolved materials.

This step was followed by addition of 1% (10 ml) penicillin-streptomycin solution. Fetal bovine serum (FBS) (Hyclone, USA) was thawed at room temperature and then heated in water bath at 56ºC for 30 minutes.

This was followed by addition of the 10% F.B.S. v/v (100 ml) to medium and then deionized water was added to make up 1000 ml as the final volume of medium. This prepared medium was sterilized by passing through a 0.22µm filter (NALGENE Company, USA) using vacuum pump in 10-20 minutes. The prepared medium container was then sealed and kept at 4ºC until use.

2.1.2 Dulbecco's Modified Eagle's Medium (DMEM)

The medium was prepared by mixing 13.65 gram Dulbecco's Modification of Eagle's Medium (DMEM) (Sigma) with 500 ml of distilled water mixed with magnetic stirrer, and was followed by adding sodium bicarbonate.

The medium was then filtered through sterilized filter with diameter of 0.22µm (NALGENE Company, USA), and was kept at 4°C. In order to make a complete medium, heat inactivated fetal bovine serum (HyClone, USA) was added to a final concentration of 10% v/v. The medium was kept at 4°C.

2.2 Staining preparation

2.2.1 Coomassie blue stain

Preparation

7 ml of acetic acid was added to 40 ml methanol, and then 53 ml of distilled water were added, mixed well and then 0.2 gm Coomassi blue powder stain added and the reagents were mixed and filtered using a whatman paper and store at room temperature.

2.2.2 Ponceau S stain

Preparation

Ponceau S stain was prepared by dissolving 0.1g of stain powder in 100 ml of 1% acetic acid.

2.2.3 Methods of haematoxylin staining

After drying the slide section at room temperature overnight, the slides (3-4-µm thickness) were fixed in cold methanol for 10 minutes, and washed with running water for