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THE PRODUCTION OF MURINE MONOCLONAL ANTIBODIES (MAb) DIRECTED AGAINST HUMAN T- LYMPHOCYTE SUBSETS

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ABSTRACT

The production of murine monoclonal antibody (MAb) has not yet been reported in Iran. The present work describes for the first time the generation of several murine hybridoma clones secreting MAbs directed against human leukocyte surface antigens. The secreted antibodies by hybridoma clones have been screened on different lymphoid and non-lymphoid tissues. Results indicated that of seven hybridomas, clones 3F11, 3C3 and 1F2 showed a strong reactivity for T-cells purified from thymus and tonsil tissues. Moreover, clones 1D4 and 6G5 which partially stained thymus tissues were found to be negative on purified B-cells and monocytic cell line U937. The third group, 4E5 and 4F4 hybridoma clones was expressed weakly on purified T, B-cells and U937 cell line. Further work is needed to determine the epitopes recognized by these MAbs and a comparison of the data with those presented at the previous leukocyte antigens workshop.
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INTRODUCTION

Over the past ten years, monoclonal antibody technology has revolutionized our knowledge concerning the physiology of lymphocytes. This approach has provided endless information about the molecular structures and determinants expressed on the lymphocytes' cell membrane as well as molecules and factors utilized during lymphocyte growth and differentiation. It is now known that most of the lymphocyte's membrane proteins are engaged in the process of cell-cell communication and adhesion.[1,2] It is also well established that MAbs to a number of B and T-lymphocyte surface antigens are capable of inducing either growth or inhibitory signals.[3-5] This is examplified by MAb to CD20, a 35 KDa antigen expressed on human B-cells which induces resting B-cells to leave the G0 and enter the G1 phase of the cell cycle.[6,7] MAb directed against CD21, a 140 KDa molecule (also known as C3d receptor) have been shown to induce B-cell proliferation, an activity which has already been ascribed to polymerised C3d on B-cells.[8] Similar activity has been reported for a MAb to CD40, a 50 KDa molecule expressed on normal malignant B-cells[6] and for MAb to CD23, a 45 KDa glycoprotein (also known as IgE low-affinity receptor) expressed on most bone marrow-derived B-cells.[9,10] In contrast, most MAb to a 95 KDa B-cell specific antigen, the CD19, was shown to down-regulate the process of B-cell activation, possibly by delivering an inhibitory signal.[11] Utilization of an activation signal following CD3, CD2, CD8, and CD28 ligation by specific MAb in case of T lymphocytes has been extensively studied.[4,5,12-14] MAb from this point of view represent a suitable pharmacological agonist to deal with conditions of disregulation of lymphocyte growth and hyperactivation, and also to better understand normal physiological pathways of cell division and its essential requirements. This, in addition to what has been done in order to use leukocyte-specific MAb as a probe for diagnosis and classification of the haemopoietic neoplasia,[15] and drug-targetting using MAb have opened a new dimension in molecular therapy of cancer. In the

current work, we report the production of seven murine monoclonal antibodies specific for human leukocyte surface antigens. These cloned hybridomas could be very useful tools for diagnosis and immunophenotyping of leukemia as well as for bone marrow purging.

MATERIALS AND METHODS

Medium and reagents

RPMI-1640 medium, heat-inactivated fetal calf serum (FCS), penicillin, and streptomycin were all obtained from Gibco, Scotland. FITC and peroxidase conjugated goat polyclonal antibodies to mouse Ig, hypoxanthine, thymidine, aminopterin, thioguanuine, polyethylenglycol, goat anti-mouse Ig sub-classes all were obtained from Sigma Co.

Cell lines and animals

Human promyelocytic cell line (U937) was kindly provided by Dr. F. Shokri, Department of Pathobiology, Faculty of Public Health, Tehran University of Medical Sciences, Tehran . The HAT sensitized mouse myeloma cell line X63.AG.8.653[16] was kindly provided by Dr. Denis. R. Stanworth, Department of Immunology, University of Birmingham, England. BALB/c mice were obtained from the animal lab of Shiraz University of Mecidal Sciences.

Generation of murine monoclonal antibodies

BALB/c mice were immunized intraperitoneally (IP) with a 20×10^6 human leukocyte suspension in saline. Repeated immunization with the same number of cells were performed on days 14, 21, and 28. The spleen cells from a hyperimmunized mouse killed by cervical dislocation were aseptically removed and placed into a sterile homogenizer tube containing 5mL of RPMI-1640 medium. The prepared cell suspension was centrifuged, followed by lysing the red cells by adding 1 mL of distilled water, the remaining leukocytes were resuspended in 10 mL RPMI-1640 medium supplemented with 10% FCS and thioguanine at a concentration of 2×10^{-5}M. Thioguanine selects cells deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPT).

Fusion protocol

The principle of fusion protocol in this work was based on the method described by Diamond, et al.,[17] in which mouse myeloma cells were mixed with mouse spleen cells at a ratio of 1:2 or 1:5 in a total volume of 20 mL of cold RPMI-1640 medium following which the cells were centrifuged at 200 g for 10 min. The supernatant was removed and the pellet resuspended in the remaining volume and 0.8 mL of warm 40% polyethylenglycol 4500 (PEG) was added to the pellet dropwise over one minute. The cells were left to stand for another one minute and 20 mL of warm RPMI-1640 medium was then slowly added over a period of 10 minutes. The mixture was centrifuged, the supernatant was removed and the pellet resuspended in 100 mL HAT medium with 20% FCS. 200 µL aliquots of the cell suspension was added to the 96-well flat bottom plates 100 µL aliquots of fresh HAT media was added to each well on day three post-fusion. Visible hybridomas were noted around days 6-10.

Table I. Reactivity of 1F2, 3C5 and 3F11 hybridoma clones with lymphoid and myeloid cells in an indirect fluorescent test.

	1F2	3C5	3F11
T-cells (thymus)	++++	++++	++++
T-cells (tonsil)	++++	++++	++++
Act. T-cells*	+	- / +	+
B-cells	++	++	++
U937	+	+	+

*Act.T-cells= 48 hours Con-A activated T-cells.

Screening of the hybridomas

Hybridoma culture supernatants from 96 well plates were washed in tubes (4 mL) and the pellet resuspended in 100 µL of hybridoma culture supernatants. The cells were incubated for 45 minutes on ice. After two washes (RPMI-1640 medium supplemented with 2% FCS and 0.01% sodium azide), 100 µL of a goat anti-mouse Ig-FITC conjugate was added to the tubes and incubated as above. The cells were washed three times with the same washing buffer and the pellet was resuspended in 0.2 mL washing buffer for examination under a Leitz fluorescent microscope.

Preparation of tonsillar high density B-cells

The B lymphocytes used in this study were separated as follows. An equal volume of 5% AET-modified sheep erythrocytes was added to the tonsillar mononuclear cells at 5×10^6 cell/mL and incubated for five min at 60 g for 3 min, incubated on ice for one hour and then spun through Ficoll Hypaque in order to remove rosetted and free red cells. This procedure was repeated. The remaining mononuclear cells were resuspended to the desired concentration and were used as B-cells fraction throughout this study.

Indirect immunofluorescence on tissue sections

Ascitic fluids or hybridoma culture supernatants were added to the frozen sections from tonsil or thymus and

Table II. Reactivity of 6G5 and 1D4 hybridoma clones for lymphoid and myeloid cells in an indirect fluorescent test

	6G5	1D4
T-cells (thymus)	++	+++
T-cells (tonsil)	++	+++
Act. T-cells*	-	-
B-cells	-	-
U937	-	-

Table III. Reactivity of 4F4 and 4E5 hybridoma clones for lymphoid and myeloid cells in an indirect fluorescent test

	4F4	4E5
T-cells (thymus)	++	+
T-cells (tonsil)	++	+
Act. T-cells*	++	+
B-cells	++	+
U937	-	-

incubated in a moist chamber at room temperature for 30 minutes. The slides were washed in a stream of PBS and then placed in a PBS bath with magnetic stirrer for 15 minutes. Excess PBS was gently removed from the slides and fluorescein-conjugated goat anti-mouse Ig antibody was applied to the slides. The slides were mounted under coverslips in DABCO and examined by the Leitz fluorescence microscope.

RESULTS

After three different fusions of mouse myeloma cell line human leukocytes, 74 different hybridomas were produced. The initial screening of these hybridomas was based on their pattern of staining for human leukocytes derived from tonsil tissues. Of 74 hybridomas tested, seven were found to be positive on purified T- and B-lymphocytes from thymus and tonsil. Immunofluorescence staining of thymus and tonsil as well as reactivity with a monocytic cell line U937 have revealed that these seven hybridomas can be categorized in three groups.

MAbs with strong reactivity on thymus and tonsil tissues

Hybridoma clones 3F11, 3C5 and 1F2 were found to react strongly with thymus and tonsil tissues. Purified T-cells and B-cells were prepared by negative selection and tested by immunofluorescence. Results indicated that although purified T-cells from both thymus and tonsil were stained intensely by these three MAbs, their pattern of reactivitieson purified B-cells was not comparable to what was reported for T-cells. Purified preparation of T-cells stimulated by Con-A for 48 hours were assayed for reactivity with these MAbs. The results showed that mitogen-induced stimulation of T-cells leads to a weak expression of 3F11, 3C5 and 1F2. Further investigation of the possible expression of epitope recognized by these MAbs was made by using a promonocytic cell line, U937 in immunofluorescence test. Data indicated that MAbs of this group demonstrated a weak pattern of staining for U937 cell line (Table I). The isotypes of 3F11 was found to be IgG1 while 1F2 and 3C5 MAbs carried IgG2a and IgM isotypes, respectively. The pattern of reactivities of these MAb is summarized in Table I.

MAbs which stain certain T-lymphocyte populations

Two different MAbs have been selected which seem to react with some subsets of T-cells, these include 1D4 and 6G5 clones. 1D4 was found to react with 80% of T-lymphocytes derived from the thymus and tonsil tissues, indicating an equal proportion of these epitopes on thymocytes and tonsillar T-cells. On the other hand, 6G5 MAb was tested on the same preparation of T-cells. The results showed that this MAb in contrast to that of 1D4 clone was able to stain only 40% of the T-cells. Purified T-cells from thymus were stimulated by Con-A for 48 hours and their reactivity for 1D4 and 6G5 MAbs were determined. The results showed that in contrast to reactivity of mitogen unprimed T-cells, the mitogen-activated T-cells failed to express 1D4 and 6G5 epitopes. Both 1D4 and 6G5 clones failed to stain B-lymphocytes prepared from tonsil tissue. Similar to antibodies described above, 1D4 and 6G5 were negative when tested on U937 cell line. The isotype of 1D4 and 6G5 MAbs were found to be IgG1 and IgG2b, respectively. The pattern of reactivities of 1D4 6G5 is summarized in Table II.

MAbs with reactivity for a subset of T-cells with sharing epitopes on B-cells

Two different MAbs named 4E5 and 4F4 were selected and their pattern of reactivity on purified T- and B-cells were investigated. 4E5 and 4F4 reacted with 40% of thymocytes, while reacting with all B-lymphocytes purified by negative selection from tonsillar mononuclear cells. Con-A stimulation of T-cells failed to up-regulate 4E5 and 4F4 epitopes but the percentage of positive cells for these two MAbs before and after mitogen stimulation were unchanged. The 4E5 and 4F4 isotypes were determined by doublediffusion test, indicating that these MAbs carried IgG2b and IgG1 isotypes, respectively. The pattern of reactivities of these MAbs is summarized in Table III.

DISCUSSION

The technology of monoclonal antibodies during the past decade has been able to successfully diversify both the theoretical and technical aspects of medicine. Even the fast growing immunological science in this period has been an inevitable consequence of this technology, implying the immunological philosophy for better understanding of the etiology and cause of diseases on the one hand, and on the other hand the practical reality for measurement and assays of trace biological by-products are worth mentioning. Drug targetting and chimeric antibody for cancer immunotherapy could well be the latest version of this powerful tool which seems to be a promising approach. Despite the fast growing biotechnology branches in the industrial world, the production of monoclonal antibody (one of the important biotechnological products) has not yet been reported in Iran. The current work describes for the first time production of seven hybridoma clones selected among the 74 different hybridomas reacting with human leukocyte surface antigens. The selection criteria was based on the reactivity of hybridoma culture supernatants with human leukocytes prepared from tonsil as well as single cell suspension of thymocytes by indirect fluorescence. According to the pattern of reactivity of hybridoma supernatants, seven were selected and classified into three groups. The first three clones secreted MAb which reacted strongly with thymus-derived lymphocytes and purified T-cells from tonsils but demonstrated a weak reaction for purified B-cells. A practical way of grouping these MAbs could be the use of different T- and B-cell lines. In our study, purified tonsillar B-cells have been used instead of different B-cell lines, where such B-cell preparations always contained a percentage of residual T-cells. This could well explain the positivity seen by these groups of MAbs on B-cell preparations. The strong reactivity of these MAbs for thymocytes clearly indicated that the epitopes recognized by these MAbs could be restricted to those determinants expressed on T-cells. The weak expression of these epitopes following mitogen stimulation of T-cells could be very important. This phenomenon has already been seen in several T-cell associated markers such as expression of different isoforms of CD45 and CD2.[18-20] The down-regulation of epitopes recognized by these MAbs following mitogen stimulation has been the case for all three clones of this group.

Regarding the 1D4 and 6G5 clones, as seen in the results section, these two MAbs recognize different subsets of T-cells without cross reaction with normal B-cells and a monocytic cell line, U937. It will soon become practicable to purify these two different subsets of T-cells from either thymus or other lymphoid tissues for more characterization and epitope mapping. It would be of great interest to compare the percentage of positivity seen by 1D4 and 6G5 with those of MAbs to CD4 and CD8 T-cell associated markers. The lack of expression of these epitopes on 48 hour mitogen-stimulated T-cells indicated that these epitopes were lost following T-cell activation. Regarding the pattern of staining of 4E5 and 4F4 clones on both T-and B-lymphocytes and U937 cell line; it is worth mentioning that several MAbs have already been reported with such properties.[20] These epitopes are known as the common leukocyte antigens. The expression of these epitopes are wide and non-lineage-specific. The best example of common leukocyte antigens are the CD45 series and CD52. [20] MAbs to the common leukocyte antigens have important applications in several respects. One of the commonest applications is the bone marrow purging in which mature leukocytes of the bone marrow preparation are depleted by these types of antibodies. Although the major criteria for grouping of these seven hybridoma clones has been the pattern of leukocyte staining, further work is needed to characterize these MAbs. Among these are the immunoblotting technique which determines the molecular weight of the antigenic determinants for clustering, comparison of these epitopes with each other and with known and clustered workshop antigens. This task is currently a major priority in our laboratory.

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Editorial Comment

This article is a primary report of monoclonal antibody production in Iran. There are many implications of monoclonal antibodies in clinical and basic medical investigations. Because of cost-effective benefits of such advanced systems in our country, we present this article to encourage our specialists in such necessary fields.

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