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An Insight to Characterize Molecules Expressed on Activated Lymphocytes of Water Buffalo

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Abstract: Analysis and elucidation of the changes in water buffalo immune response to infectious agents and their derived vaccines, is largely associated with limited progress due to lack of monoclonal antibodies (mAbs) that recognize molecules expressed on the surface of their immune cells. The aim of the present study was to provide an insight toward the immune system of such species by determining the expression of certain molecules that are expressed on activated lymphocytes. Blood was collected from apparently healthy animals (4 adult animals of 2-3 years old). Peripheral blood mononuclear cells (PBMCs) were recovered and cultured in the presence of concanavalin A (ConA) for 48 hours. Lymphocytes were harvested, labeled by mAbs that are known to identify molecules upregulated on bovine lymphocytes and analyzed by flow cytometry. Initial screening of these mAbs-specific molecules identified those that recognize CD25, ACT1, ACT2, ACT9, ACT16, ACT27, ACT28, ACT29, ACT30, ACT31, ACT32, ACT33, ACT34, ACT35, ACT36, ACT37, ACT38; which were upregulated only on activated lymphocytes and not on resting cells. In conclusion, identification of these mAbs-specific molecules could be useful in analyzing the immune response of water buffalo to infectious agents and their derived vaccines. Future studies are needed to investigate the functional roles of these molecules during the development of the immune response.

Key words: Water Buffalo · Activation Molecules · Monoclonal Antibodies · Flow Cytometry

INTRODUCTION

Water buffalo is an essential component of the animal wealth in many countries including Egypt for their meat and milk production. Water buffalo are the second largest source of milk supply that contains less water and more fat, lactose and protein than cow milk) [1]. Infectious diseases of water buffalo are managed like those of cattle as both species are susceptible to a similar spectrum of infectious microorganisms. Many vaccines and therapeutics that were developed to be used for control of infectious agents among cattle [2, 3]; have proven to be effective in water buffalo treatment and management although both species respond to certain infectious agents differently [4]. The reason for the use of cattle control programs among water buffalo was suspicion that the theories regulating the development and expression of immune response have been based primarily on findings from studies conducted in cattle and the assumption that the immune system and immune response among ruminant animals; is basically similar, but recent immunological findings suggest that this approach must be reconsidered [5].

To evaluate the immune response of water buffalo to infectious agents and potential vaccines, it is necessary to characterize their immune system and elucidate the changes in immune response that account for the development of protective immunity. This necessitates the identification of molecules that are expressed on the surface of lymphocytes upon their activation, as these molecules should prove playing a vital role in the development of protective immunity to infectious agents and their derived vaccines [6-9]. So, the present study was conducted to investigate the molecules expressed on activated lymphocytes of water buffalo using monoclonal antibodies (mAbs) and flow cytometry.

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This was achieved by screening several mAbs, previously verified to recognize bovine lymphocyte activation (ACT) molecules; to determine their cross reactivity and ability to recognize corresponding molecules in water buffalo.

MATERIALS AND METHODS

Animals: Healthy adult water buffalo (n= 4) of 2-3 years old were used in this study. From each animal, 500 ml of blood was collected via jugular vein venepuncture in acid citrate dextrose containing glass bottle (ACD working solution/liter: 24.5 g dextrose monohydrate; 22.0 g sodium citrate dihydrate and 7.3 g citric acid anhydrous; pH adjusted to 7.0-7.3; filtered through a 0.2µ disposable filter). After blood collection, samples were sent to the laboratory with a minimum of delay and processed within four hours [10].

Monoclonal Antibodies (mAbs): The mAbs used in this study were obtained from monoclonal antibody center, Washington State University, USA. The specificity and isotype of these mAbs are listed in Table 1. These mAbs were developed by standard methods and the specificity of these mAbs has been verified by studies conducted in their laboratory and collaborative studies conducted during ruminant's leukocyte differentiation antigen workshops. The stock mAbs were stored at -20°C until being diluted to working dilution 15 µg/ml [7, 8, 11-14].

Fluorescein conjugated goat antimouse polyclonal IgG and IgM: It was obtained from Invitrogen Life Technologies and was used as s second step reagent for staining of labeled cells with mAbs at a working dilution 1:200 [7].

Detection and analysis of molecules expressed on resting cells (T0 labelling): For preparation and recovery of leukocytes for detection molecules expressed on resting cells, the collected blood was lysed in Tris-buffered ammonium chloride to remove red blood cells (Tris-NH4CL working solution/liter: 8.7 g NH4Cl and 1.21 g Tris base; pH adjusted to 7.2-7.4 and sterilized by autoclaving) and cells were harvested by centrifugation at 1500 RPM. The cell pellet were washed thrice with phosphate buffered saline (PBS working solution/liter: 7.65 g Sodium chloride; 1.2688 g Disodium phosphate; 0.1 g Monosodium phosphate and 0.211 g Monopotasium phosphate; pH adjusted to 7.2-7.4 and sterilized by autoclaving) containing 20% ACD by centrifugation at 1000 RPM to remove excess platelets.

Table 1: List of mAbs used in this study, their isotype, specificity and reactivity with water buffalo ConA activated lymphocytes.

mAb	Ig Isotype	Specificity	Reactivity with Water buffalo lymphocytes
CACT116A	G1	CD25[b]	+
CACT260A	M	CD25[b]	+
LCTB2A	G3	CD25[b]	+
GB112A	G1	CD25[g]	+
CACT177A	G1	ACT1	+
CACT200A	G1	ACT1	+
CACT206A	G2a	ACT1	+
CACT276A	G2a	ACT1	+
CACT292A	M	ACT1	+
CACT298A	G2a	ACT1	+
CACT26A	G1	ACT2	+
LCTB6A	G1	ACT9	+
GB110A	M	ACT16	+
CACT195A	M	ACT27	+
CACT216	M	ACT28	+
CACT225A	G1	ACT29	+
CACT185A	G1	ACT30	+
CACT152A	M	ACT31	+
CACT191A	M	ACT32	+
CACT164A	M	ACT33	+
CACT180A	G1	ACT34	+
CACT282A	M	ACT35	+
CACT295A	G1	ACT36	+
CACT153A	M	ACT37	+
CACT243A	M	ACT38	+

A plus (+) sign indicates a mAb recognizes a conserved epitope on an orthologous molecule. ACT = molecule expressed on activated lymphocytes.

After the last wash, cell pellet was resuspended in first wash buffer (PBS containing 0.01% w/v Na azide, 10% v/v ACD and 0.02% v/v horse serum) and cell viability was determined by trypan blue exclusion test and kept on ice until being used for labeling for flow cytometry [10].

Detection and Analysis of Molecules Expressed on Activated Cells: For detection and analysis of molecules upregulated on activated cells, fresh collected blood in ACD was centrifuged at 1500 RPM for 30 minutes and the buffy coat layer was harvested. The harvested cells were then subjected to density gradient centrifugation at 1500 RPM for about 30 minutes using Accu-Paque (density, 1.086; Accurate Chemical and Scientific Corp., Westbury, N.Y.). Residual red blood cells in the buffy coat were lysed in Tris-NH4CL. PBMCs were collected and washed thrice in PBS-ACD by centrifugation at 1000 RPM for 10 minutes to remove excess platelets. The recovered cells were cultured at concentration 1-2 × 10^6 cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 µM L-glutamine, 1mM sodium pyruvate, 20 IU/ml of penicillin and 20 µg/ml of streptomycin, 50 µM 2-mercaptoethanol (SIGMA) and ConA (5 µg/ml) and incubated at 37°C in a 5% CO_2 incubator for 48 hours. At the end of incubation, cultured cells were harvested and cell viability was determined by trypan blue exclusion test and kept on ice until being used for labeling for flow cytometry [15, 16].

Labeling of Cells: All primary monoclonal antibodies (mAbs) used in the present study are listed in Table 1. For labeling of cells with mAbs, One hundred µl of cell suspension containing one million viable cells were distributed in 96 well V-shaped bottom microtiter plate containing 50 µl of mAb (15 µg/ml) and incubated in the dark for 30 minutes on ice. Cells were subjected to 3 cycles of washing at 2000 RPM for 2 minutes using first wash buffer. After the final wash, the cell pellet was resuspended in 100 µl of second step reagent (FITC goat antimouse polyclonal IgG and IgM) and incubated in the dark for 30 minutes on ice. Cells were subjected to 2 cycles of washing at 2000 RPM for 2 minutes using second wash buffer (PBS containing 0.01% w/v Na azide and 10% v/v ACD). After the final wash, cell pellet was resuspended PBS-buffered formaldehyde (PBS containing 2% v/v formaldehyde) and kept in the dark at 4°C until being analyzed by flow cytometry [10, 17, 18].

Collection of Cells by Flow Cytometry: Collection of cells by flow cytometry was done by BD FACSCalibur flow cytometer equipped with argon and red lasers, a Macintosh 5 computer, equipped with Cell Quest software. FCS express software was used to analyze the data.

RESULTS

Strategy for Collecting and Analyzing Flow Cytometric Data: At time of cells collection, forward and side light scatter parameters (FSC vs. SSC) were used to distinguish granulocytes from mononuclear cells (monocytes and lymphocytes). Two gates were placed on mononuclear cells to distinguish lymphocytes (Gate 1) from monocytes and large lymphocytes (Gate 2). Cells in the gates were

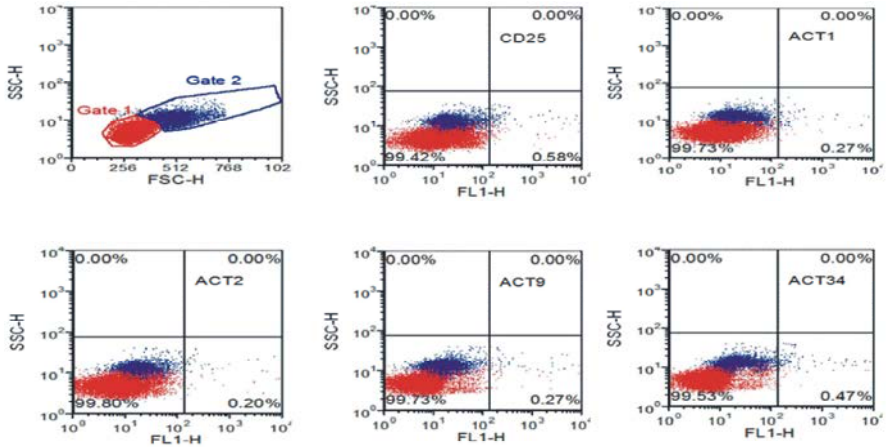

Fig 1: Representative dot plot profiles of resting PBMCs labeled at T0 with the indicated mAbs. A single representative profile is shown for mAbs that recognize the same molecule on the same subset of cells. A side light scatter (SSC) vs. forward light scatter (FSC) dot plot was used to gate and color code PBMCs: red for lymphocytes and blue for monocytes. The profiles indicate no expression of the indicated molecule on resting cells.

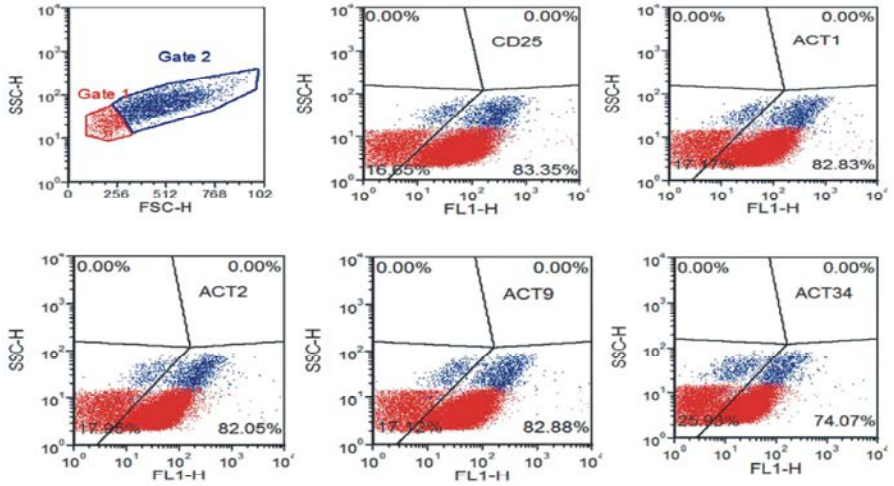

Fig 2: Representative dot plot profiles of harvested cells following 48 hours activation by ConA and labeled with the indicated mAbs. A single representative profile is shown for mAbs that recognize the same molecule on the same subset of cells. A side light scatter (SSC) vs. forward light scatter (FSC) dot plot was used to gate and color code cells: red for small lymphocytes and blue for large ones. The profiles indicate expression of the indicated molecule on ConA activated cells.

artificially colored coded for analysis and distinction. The two gates were used to exclude granulocytes, dead cells and debris. The FC was instructed to collect 25000 events (cells) within the gated events (R1 or R2). Quadrant markers were placed on the analysis dot plot (SSC vs. FL1 parameters) for each mAb to show the negative and positive populations and the expression pattern of their molecules (Fig. 1 and Fig. 2).

Identification of mAbs Recognizing Activation Molecules (ACT) Expressed on ConA Activated Cells: The mAbs reactive with molecules expressed on ConA activated lymphocytes are summarized in Table 1. Initial screening of these mAbs-specific molecules identified those that recognize CD25, ACT1, ACT2, ACT9, ACT16, ACT27, ACT28, ACT29, ACT30, ACT31, ACT32, ACT33, ACT34, ACT35, ACT36, ACT37 and ACT38. All these mAbs cross reacted with water buffalo activated cells similar to those in cattle.

DISCUSSION

Two particular immunological techniques allowed the research in immunity and its disorders in human and animals; the development of mAbs that could identify surface leukocyte differentiation molecules (LDM) [19] and the flow cytometry [20]. Both are used now to fully characterize the immune system and analyze the immune response of human and animals against infectious agents and their derived vaccines.

Previous studies revealed the specificity of mAbs developed against surface MHC and LDM can be determined using flow cytometry. The mAbs that recognize epitopes on the same molecule yield the same pattern of labeling and form clusters [7]. Because of variation in the level of expression of a given molecule on one or more population of leukocytes, the patterns of labeling obtained with mAbs that recognize different molecules are unique. This observation has been used to cluster mAbs that recognize different leukocyte molecules [21, 22]. Further studies have shown that the patterns of expression of orthologous molecules are conserved among the different species [6, 7]. In addition, the more closely related the species are, the greater the possibility that mAbs developed against LDM molecules in one species would recognize an epitope conserved on an orthologous molecule in other species as cattle, goat, sheep [8, 23] and water buffalo [6, 9].

As demonstrated in this study, the pattern of molecules upregulated on activated lymphocytes is similar to that found in cattle following stimulation with ConA [24]. All the tested mAbs that were generated against ACT molecules expressed on activated cattle lymphocytes; were shown to recognize epitopes conserved on orthologous molecules in water buffalo.

This includes mAbs that recognize CD25, ACT1, ACT2, ACT9, ACT16, ACT27, ACT28, ACT29, ACT30, ACT31, ACT32, ACT33, ACT34, ACT35, ACT36, ACT37 and ACT38 molecules. These mAbs recognized conserved epitopes on orthologous ACT molecules and should prove useful in future studies for analyzing the immune response of water buffalo to infectious agents and their derived vaccines.

CD25 (IL-2R) is a marker of activation, expressed on activated cells including T and B cells within 16-24 hours of ConA stimulation [24]. The CD25 subunit of the human IL-2R is also present on a subset of thymocytes. IL-2 induces the expression of the CD25 subunit on NK cells. CD25 contains an α-chain which is a low-affinity IL-2 receptor and a β-chain which is a medium-affinity receptor and an activation marker which induces the activation and proliferation of T cells, thymocytes, NK cells, B cells and macrophages. $CD4^+CD25^+$ regulatory T cells seem to regulate other T cells and play a role in limiting inflammatory responses and preventing autoimmunity as knockout mice develop autoimmune diseases [25].

ACT1 is expressed within 16-24 hours of ConA stimulation similar to kinetics of appearance of CD25. ACT2 is expressed on $WC1^+$ cells. Its time of expression varies, but, it is prominently expressed following 5 days of ConA stimulation. It is expressed on $CD8^+$ cells in the intestine and mammary gland [26]. ACT3 is differentially expressed on $\alpha\beta$ and $\gamma\delta$ T cells. It is predominantly expressed on $CD4^+$ cells following stimulation with ConA following 5 days of culture. In long term cultures maintained on IL-2, ACT3 appears on $\alpha\beta$ and $\gamma\delta$ T cells. ACT3 appears on $CD8^+$ cells following stimulation with the superantigen staphylococcal enterotoxin C [27]. ACT13 and ACT14 are prominently expressed on B cells following stimulation of PBMCs with ConA. ACT16 is expressed on a variable number of lymphocytes following ConA stimulation. ACT17 is expressed on all T cells following 24-48 hours. The kinetics of appearance differs from those of ACT1 and CD25 [24]. No data are available on the kinetics of expression of other ACT molecules. Future studies are needed to investigate the expression of these molecules on different lymphocyte subpopulations and their functional roles during the development of the immune response.

In conclusion, the present study succeeded in identifying mAbs that recognize orthologous ACT molecules in water buffalo. In future studies, these mAbs could be useful in characterizing and analyzing the immune response of water buffalo to infectious agents and their derived vaccines.

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Conflict of Interest: All authors declare that they have no conflict of interest on any data published in this manuscript.

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