

The use of crossreactive monoclonal antibodies to characterize the immune system of the water buffalo (*Bubalus bubalis*)

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One of the major difficulties in studying the mechanisms of host defense in economically important species indigenous to Asia and the Middle East is the lack of monoclonal antibody (mAb) reagents that define the immune systems of species other than cattle, goats, sheep, and pigs. One strategy that could obviate this problem at minimal cost is to identify existing mAbs that recognize conserved epitopes on orthologous major histocompatibility complex (MHC) and leukocyte differentiation molecules. To explore the potential of this approach, we screened a large set of mAbs that recognize bovine MHC class I and II molecules and leukocyte differentiation molecules to identify mAbs that react with orthologous molecules in water buffalo. One hundred thirty eight were found that recognize conserved determinants on orthologous molecules. In addition to identifying a useful set of reagents, the study has provided insight into the composition of the immune system of the water buffalo (*Bubalus bubalis*).

Key words: Water buffalo, *Bubalus bubalis*, monoclonal antibodies, leukocyte differentiation molecules, major histocompatibility complex

Introduction

The water buffalo (*Bubalus bubalis*) is an essential component of Asian agriculture, representing over one quarter of all domestic Asian bovids [11]. In some nations, the majority of domestic bovids are water buffalo (Philippines, 63.0%; Laos, 62.9%; Thailand, 56.2%). They are used as draft animals, provide milk (52.2% of all ruminant-produced milk in the developing Far East {[11]})

and meat in regions with limited animal protein resources. Although under-utilized outside of Asia, the water buffalo has become central to Indian and Egyptian dairies and the Italian cheese industries, and serves as a minor, but growing source of meat in other nations [11,22].

Infectious diseases of water buffalo are currently managed much like those of cattle. Water buffalo and cattle are susceptible to a similar spectrum of infectious agents, and many of the vaccines and chemotherapeutic regimens developed for use in cattle have proven effective in water buffalo (examples include brucellosis, leptospirosis, anthrax, rinderpest and foot-and-mouth disease [22,24,28]). However, the health needs of water buffalo, especially young animals, are distinct from those of cattle. Wallowing behavior exposes buffalo calves to water-borne pathogens not normally encountered by cattle, and increases contact with arthropod vector-borne diseases. In addition, young buffaloes are often malnourished, resulting from the owner's use of milk from nursing animals. Finally other, potentially genetically based differences exist between cattle and water buffalo. These currently undefined differences are reflected in water buffalo as a heightened susceptibility to diseases such as pasteurellosis, blackleg and echinococcosis. Some agents, such as buffalo poxvirus, are apparently unique to the water buffalo [22].

Because cattle and water buffalo respond to certain infectious agents differently and often dwell in very different environments, it is likely that the needs of water buffalo will not always be met by vaccines and management regimens designed for cattle. To successfully adapt bovine-based strategies of health management to water buffalo, and to address problems unique to the water buffalo, it will be necessary to understand the variables involved in differential disease susceptibility. Such knowledge will not only contribute to improvement in the management of water buffalo, but will also provide insight into the mechanisms accounting for differences in

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resistance to diseases of economic importance to cattle such as anaplasmosis and babesiosis [25,25,26].

To evaluate the immune responses of water buffalo to infectious agents and potential vaccines, it is necessary to characterize the immune system of the water buffalo and elucidate the changes in the immune response that account for the development of protective immunity. To achieve this goal in cattle, we developed an extensive set of monoclonal antibodies (mAbs) to leukocyte differentiation molecules that are differentially expressed on one or more lineages of leukocytes in cattle [6,7] (and unpublished). The specificity of many of the mAbs has been validated by studies performed as part of international workshops held over the past few years [15,16,21]. We have hypothesized that some of the mAb developed in cattle recognize evolutionarily conserved determinants present on orthologous molecules in species other than the original target species used to make the mAbs [7]. In the present study, we screened a set of anti-bovine mAbs developed in our laboratory and also mAbs submitted to the first international workshop on ruminant leukocyte differentiation antigens [15] for cross-reactivity with MHC and leukocyte differentiation molecules of water buffalo. Monoclonal antibodies were identified that recognize major histocompatibility complex (MHC) class I and II molecules and leukocyte differentiation molecules expressed on one or more types of leukocytes in the water buffalo. These mAbs have provided an opportunity to characterize and compare the immune systems of cattle and water buffalo.

Materials and Methods

Animals: Four adult and four young water buffalo, from a herd maintained by Dr. H.L. Popenoe at the University of Florida (Gainesville, FL) served as a source of peripheral blood used in this study.

Monoclonal antibodies: The mAbs used in this study (Table 1 and not shown) were developed by standard methods and have been described elsewhere [13]. The specificity of most of these mAbs have been verified by studies conducted in our laboratory [4,7] and collaborative studies conducted during 3 ruminant leukocyte differentiation antigen workshops [15,16,21]. Some mAbs recognize molecules that have not yet been fully characterized.

Cell preparation and flow cytometry: Peripheral blood was obtained from animals by jugular venipuncture, collected into acid citrate dextrose (ACD) to a final concentration of 15-20% ACD, and shipped by overnight mail at ambient temperature. For general flow cytometry, blood was centrifuged at $500 \times g$ for 30 minutes to remove plasma, then resuspended in Tris-buffered ammonium chloride (NH_4Cl , 0.87% w/v, pH 7.4) to lyse erythrocytes.

Following one wash in phosphate buffered saline (PBS), the cells were resuspended in PBS containing 10 mM EDTA, 0.1% (w/v) Na azide, 10% (v/v) ACD and 2% (v/v) γ -globulin-free horse serum (PBS-HS [GIBCOBRL, Grand Island, NY]), 2×10^7 cells/ml. Cells were then distributed in 50 μl aliquots to wells of 96-well V-bottom microtiter plates containing either 50 μl of PBS-HS (negative control) or 1 μg of mAb in 50 μl of PBS-HS and incubated for 30 minutes at 4°C . Cells were washed 3 times ($800 \times g/3$ min) in PBS-HS, then incubated in the dark for 30 minutes at 4°C in 100 μl of a 1:200 dilution of fluorescein (FITC)-conjugated goat anti-mouse immunoglobulin (IgG and IgM specific {Caltag Laboratories; Burlingame, CA}). Cells were washed twice as above in PBS, then fixed for 30 minutes in 2% (v/v) PBS buffered formaldehyde. For analysis of activated cells, blood was centrifuged at $500 \times g$ for 30 minutes and the buffy coat layer of cells harvested. Cells were then subjected to density gradient centrifugation ($600 \times g$ for 20 minutes) using Lympho-paque (density 1.086; Nycomed AS; Oslo, Norway). Mononuclear cells banding at the interface were collected and washed once ($500 \times g/10$ min) in phosphate-buffered saline (PBS, pH 7.2), then incubated with NH_4Cl to lyse residual erythrocytes. The cells were resuspended in Dulbeccos modified Eagle medium (DMEM) containing 13% calf bovine serum (CBS), glutamine, and antibiotics and cultured with concanavalin A (Con A, 5 $\mu\text{g}/\text{ml}$) for 48 hr. The cells were then labeled for flow cytometry, fixed, and kept at 4°C until analyzed. Cells were analyzed on a FACSCAN flow cytometer using LYSYS and Cell Quest software (Becton Dickinson Biosciences, San Jose, CA) [2].

Results

Monoclonal antibodies reactive with leukocytes in water buffalo

The data are summarized in Tables 1 and 3. mAbs reactive with molecules expressed on resting cells were screened on unseparated preparations of leukocytes. mAbs known to react with molecules upregulated or only expressed on activated cells were screened on Con A blasts. All mAbs showing reactivity with water buffalo leukocytes were examined further to determine if the pattern of reactivity of the mAbs with water buffalo leukocytes was similar or identical to the pattern of reactivity with bovine leukocytes. mAbs that exhibited weak reactivity were considered to recognize a related epitope with lower affinity or an unrelated epitope. mAbs that exhibited a different pattern of reactivity were considered to react with an unrelated molecule. mAbs with these patterns of reactivity were not evaluated further. Initial screening of over 200 mAbs yielded 138 with patterns of reactivity similar or identical to those in cattle.

Table 1. Monoclonal antibodies reactive with bovine and water buffalo MHC and leukocyte differentiation molecules.

mAb	Ig isotype	Specificity
H1A	IgG2a	MHC class I
H6A	IgG2a	MHC class I
H58A	IgG2a	MHC class I
PT85A	IgG2a	MHC class I
B5C	IgG2b	MHC class I
TH14B	IgG2a	MHC class II (HLA-DR orthologue)
TH81A5	IgG2a	MHC class II (HLA-DQ orthologue)
H42A	IgG2a	MHC class II (HLA-DP orthologue?)
BAQ95A	IgG1	CD2
MUC2A	IgG2a	CD2
CH128A	IgG1	CD2
CH134A	IgG1	CD2
CACT31A	IgM	CD2
CACT98A	IgM	CD2
BAT18A	IgG1	CD2
BAT42A	IgG1	CD2
BAT76A	IgG2a	CD2
LCTB36A	IgG1	CD2
MM1A	IgG1	CD3
CACT93B	IgM	CD4
CACT138A	IgG1	CD4
GC50A	IgM	CD4
ILA11A	IgG2a	CD4
CC17	IgG1	CD5
BAQ82A	IgM	CD6
BAQ91A	IgG1	CD6
CACT141A	IgG2b	CD6
BAQ111A	IgM	CD8 α
TH82D1	IgG1	CD8 β ?
CACT80C	IgG1	CD8 α
CACT88C	IgG3	CD8 α
7C2B	IgG2a	CD8
ST8	IgM	CD8
GB21A	IgG2b	$\gamma\delta$ TCR (δ chain specific)
CACT18A	IgM	$\gamma\delta$ TCR (δ chain specific)
CACT19A	IgM	$\gamma\delta$ TCR-N6 cluster
GB22A	IgG1	$\gamma\delta$ TCR-N6 cluster
CACTB6A	IgM	$\gamma\delta$ TCR-N6
CACTB14A	IgG1	$\gamma\delta$ TCR-N6 cluster
CACTB81A	IgG1	$\gamma\delta$ TCR-N7
CACTB44A	IgG1	$\gamma\delta$ TCR-N7 cluster
CACT22A	IgM	$\gamma\delta$ TCR-N7 cluster
86D1	IgG1	$\gamma\delta$ TCR-N7 cluster
B7A	IgM	WC1-N1 pan*
BAQ4A	IgG1	WC1-N2 pan
CACTB31A	IgG2b	WC1-N22 pan
GB54A	IgG2a	WC1-N25 pan
CACTB32A	IgG1	WC1-N3 subset
CACTB1A	IgG1	WC1-N3 subset cluster

Table 1. Continued.

mAb	Ig isotype	Specificity
CACTB7A	IgG1	WC1-N3 subset cluster
CACTB15A	IgG1	WC1-N3 subset cluster
BAQ76A	IgG1	WC1-N3 subset cluster
BAQ89A	IgG1	WC1-N4
BAQ159A	IgG1	WC1 N4 subset cluster
BAS2A	IgG1	WC1-N4 subset cluster
BAS3A	IgG1	SigM
BAS9A	IgM	B**
BAQ44A	IgM	B
BAQ155A	IgG1	B
LCTB16A	IgG1	B
PIG45A2	IgG2b	SigM
BIG73A	IgG1	SigM
BIG501E	IgG1	λ light chain
BIG43A	IgG1	κ light chain
RH1A	IgG3	CD9 (predicted specificity)
TH2A	IgG2a	CD9 (predicted specificity)
B18A	IgG3	CD9 (predicted specificity)
MM12A	IgG1	CD11b
MM13A	IgG1	CD11b
BAQ153A	IgM	CD11c
BAQ30A	IgG1	CD18
BAT75A	IgG1	CD11a/CD18
FW4-101	IgG1	CD29
BAG40A	IgG3	CD44
BAT31A	IgG1	CD44
CACTB51A	IgG2a	CD45
GB58A	IgG1	CD45R
CAPP2A	IgG1	CD41 platelets
GB84A	IgG1	CD42d platelets
218	IgG1	CD49d
BAQ92A	IgG1	CD62L
LCTB19A	IgG1	Pan lymphocyte
LCTB39A	IgG2a	Same as LCTB19A
TH1A	IgM	Pan lymphocyte (WC7)
TH18A	IgG3	Pan lymphocyte (WC7)
CH138A	IgM	Granulocytes
MM20A	IgG1	Granulocytes (specificity same as CH138A)
DH59B	IgG1	Granulocytes, monocytes, macrophages
TH17A	IgM	Pan leukocyte, rbc, and platelets
BAGB27A	IgG1	Pan leukocyte, rbc, and platelets
ANA8A	IgG1	WC15 rbc
CACT7A	IgM	Activation antigen 1
CACT26A	IgG1	Activation antigen 2
CACT77A	IgM	Activation antigen 2
CACT114A	IgG2b	Activation antigen 3 (WC10)
CACT116A	IgG1	CD25
GB112A	IgG1	CD25
LCTB2A	IgG3	CD25

Table 1. Continued.

mAb	Ig isotype	Specificity
LCTB28A	IgG2a	Activation antigen 13
LCTB50A	IgG2a	Activation antigen 14
GB110A	IgM	Activation antigen 16
GB127A	IgM	Activation antigen 17

*WC1 = workshop cluster 1, **B = B cells. Only representative mAbs specific for determinants expressed on all or subsets of WC1 are shown.

Table 2. Frequency of leukocyte subpopulations in peripheral blood of young and old water buffaloes

Molecule	Young animals (n = 4)		Adult animals (n = 4)	
	Mean	St Dev	Mean	St Dev
MHC class II	26	9	35	7
CD4	13	1.2	27	5
CD8	6	1.2	13	5
CD2	24	2.1	43	10
CD6	21	1.8	38	11
WC1-N1	30	9	4	1.6
WC1-N3	11	2.2	3	1.2
WC1-N4	20	3.4	2	2.1
sIgM	32	10	16	6
λ light chain	26	10	16	4
κ light chain	11	5	7	0.6
Monocytes	5	2.2	16	9

Eleven of the mAbs recognized molecules expressed on Con A activated lymphocytes. mAbs that recognized the same molecule formed clusters consistent with their recognizing the orthologous molecule in water buffalo. Others, where only one mAb showed cross reactivity, the patterns of labeling were similar or identical to the patterns of expression in cattle, consistent with the mAb recognizing the orthologue in water buffalo. This included mAbs that recognize MHC class I and II molecules, CD2, CD3, CD4, CD5, CD6, CD8, CD11b, CD11c, CD18, CD25, CD29, CD44, CD45, CD45R, CD41, CD42d, CD49d, and CD62L. Of special interest, mAbs were identified that react with the $\gamma\delta$ T cell receptor and workshop cluster 1 (WC1) that distinguish the subsets of $\gamma\delta$ T cells in cattle. Additional mAbs recognized molecules with no, as yet, identified orthologue in humans.

Distribution of lymphocyte subpopulations in young and adult water buffalo

Following identification of the cross reactive mAbs, a study was conducted with 4 young and 4 adult water buffalo to determine whether the frequency of lymphocyte subsets was similar to that observed in cattle. As in cattle, the WC1⁺ population of $\gamma\delta$ T cells was comprised of subsets that express the WC1-N3 and WC1-N4 isoforms.

The frequency of WC1⁺ $\gamma\delta$ T cells was high in young animals and low in adults. There were corresponding differences in the frequency of CD2⁺ $\alpha\beta$ T cells in young and adult animals. There was no apparent correlation in the frequency of B cells with the frequency of WC1⁺ $\gamma\delta$ T cells in young and adult animals.

Discussion

Cumulative studies of MHC and leukocyte differentiation molecules over the past few years have revealed the antigenic composition of orthologous molecules has been conserved along with patterns of expression and function. The more closely related the species are, the greater the probability that mAbs developed against molecules in one species will recognize an epitope conserved on an orthologue in other species such as cattle, goats, sheep [3,7], and water buffalo. As demonstrated here and at the third international workshop on ruminant leukocyte differentiation antigens [10,29], many of the mAbs developed against bovine MHC and leukocyte differentiation molecules react with epitopes conserved on orthologous molecules in water buffalo. mAbs have been identified that react with MHC class I and class II molecules and molecules that define the major subsets of leukocytes. mAbs have also been identified that recognize CD25 and other molecules upregulated on activated lymphocytes. Comparison of the patterns of expression of MHC and leukocyte differentiation molecules by FC has shown the patterns of expression of orthologous molecules in the water buffalo are very similar or identical to the patterns of expression in cattle. This provides further support for the supposition that the mAbs reported here indeed recognize orthologous molecules in water buffalo.

Analysis of leukocytes in peripheral blood of young and adult animals has revealed the composition of leukocyte populations in water buffalo is similar to the composition in cattle. One of the unique features to emerge from the study of the immune system of cattle is the presence of two complex subpopulations of $\gamma\delta$ T cells, one subpopulation that is similar to $\gamma\delta$ T cells described in humans and other species and a second subpopulation that has only been identified in suborders of Artiodactyla, Ruminantia,

Suiformes, and Tylopoda [1,5,9]. The two subpopulations differ phenotypically, in usage of V_{γ} , J_{γ} , C_{γ} , and V_{δ} gene segments, and in tissue distribution [1,14]. The first subpopulation is $CD2^+$, $CD3^+$, $CD5^+$, and $CD6^+$. A subset of this subpopulation is $CD8^+$. This subpopulation is low in frequency in peripheral blood (~5%) and most secondary lymphoid tissue (~5%) but high in the spleen, (~35%) [18,32]. The second subpopulation is $CD2^-$, $CD3^+$, $CD5^+$, $CD6^-$. This population is also positive for two lineage restricted molecules WC1 [18,20] and GD3.5 [17]. Limited information is available on GD3.5. WC1 is a member of the scavenger receptor cysteine rich family of molecules that includes CD5, CD6, and CD163 [30,31]. Multiple copies of the gene encoding WC1 are present in the bovine genome. Two genes have been identified that encode two isoforms that are expressed on mutually exclusive subsets of $WC1^+$ cells [1,19]. In contrast to the $WC1^-$ subpopulation, the $WC1^+$ population is present in high frequency in peripheral blood (~20-40%) of young animals. The proportion of $WC1^+$ cells decreases with age. The frequency of $WC1^+$ cells is low in lymphoid tissues (~5-10%) of young and adult animals. The frequency of these two subpopulations in peripheral blood of young and adult water buffalo compares with that seen in cattle. MAbs were identified that recognize epitopes expressed on the majority of $WC1^+$ cells and also epitopes expressed only on the N3 or N4 isoforms of WC1. These findings suggest that the distribution of $WC1^-$ and $WC1^+$ cells will be similar in lymphoid tissues to that of cattle also.

No difference was noted in the ratio of $CD4^+$ and $CD8^+$ T cells (~ ratio of 2) in peripheral blood from young and adult animals available in this study; however, the frequency of both populations was higher in adult animals. This difference was associated with a difference in the frequency of $WC1^+$ cells in young and adult animals (Table 2).

The pattern of expression of molecules upregulated on activated lymphocytes is also similar to that noted in cattle following stimulation with Con A [8]. mAbs that identify these molecules should prove useful in study of the response to infectious agents and vaccines [8]. ACT1 is expressed within 16 to 24 hrs of stimulation with con A similar to the kinetics of appearance of CD25. ACT2 is expressed on $WC1^+$ cells. Its time of appearance varies, but it is prominently expressed following 5 days of stimulation with Con A. It is constitutively expressed on $CD8^+$ cells in the intestine and mammary gland [23] (and unpublished). ACT3 (WC10) [27] is differentially expressed on ab and gd T cells. It is predominantly expressed on $CD4^+$ cells following stimulation with Con A. Expression is strong following 5 days of culture. In long term cultures maintained on IL-2 or conditioned medium, ACT3 appears on $\alpha\beta$ and $\gamma\delta$ T cells. ACT3 appears on $CD8^+$ cells following stimulation with the superantigen

staphylococcal enterotoxin C [12]. ACT13 and ACT14 are prominently expressed on B cells following stimulation of PBMC with Con A. ACT16 is expressed on a variable number of lymphocytes following stimulation with Con A. The frequency of positive cells increases with time in culture. ACT17 is expressed on all T cells following 24 to 48 hr stimulation. The kinetics of appearance differ from those of ACT1, and CD25 [8].

In summary, the results obtained in the present study show that it should be possible to obtain most of the mAbs needed for research in water buffalo from existing sets of mAbs developed in other related species. With the mAbs identified here, it will now be possible to characterize the immune system of the water buffalo and begin to analyze the immune response to infectious agents and parasites.

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