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Characterization of leukocyte subsets in buffalo (*Bubalus bubalis*) with cross-reactive monoclonal antibodies specific for bovine MHC class I and class II molecules and leukocyte differentiation molecules

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ABSTRACT

Although buffaloes (*Bubalus bubalis*) are a major component of the livestock industry worldwide, limited progress has been made in the study of the mechanisms regulating the immune response to pathogens and parasites affecting their health and productivity. This has been, in part, attributable to the limited availability of reagents to study immune responses in buffalo. As reported here, a set of cross-reactive monoclonal antibodies (mAbs), developed against bovine, ovine and caprine leukocyte differentiation molecules (LDM) and major histocompatibility complex (MHC) molecules, were identified and used to compare expression of LDM in Italian and Egyptian buffalo. The results show most of the epitopes identified with the mAbs are conserved on LDM and MHC I and II molecules in both lineages of buffalo. Comparison of the composition of lymphocyte subsets between buffalo and cattle revealed they are similar except for expression of CD2 and CD8 on workshop cluster one (WC1) positive γδ T cells. In cattle, CD8 is expressed on a subset of CD2+/WC1- γδ T cells that are present in low frequency in blood of young and old animals, whereas, CD8-/CD2-/WC1+ γδ T cells are present in high frequency in young animals, decreasing with age. In the buffalo, CD2 is expressed on a subset of WC1+ γδ T cells and CD8 is expressed on all WC1+ γδ T cells. The availability of this extensive set of mAbs provides opportunities to study the immunopathogenesis of pathogens and parasites affecting the health of buffalo.

1. Introduction

Buffaloes are an essential component of the livestock industry in many countries including Italy and Egypt. They are used as draught animals and also as a major source of milk and meat products (Borghese, 2005; Council, 1981; Wikipedia, 2014). Although considerable progress has been made in defining the genome of buffalo and in improving methods for selective breeding to enhance performance (Michelizzi et al., 2010), progress has been constrained on management of animal health. This is attributable to the limited information available on the mechanisms regulating the immune response to pathogens and parasites (Borghese, 2005; Council, 1981). Infectious diseases of water buffalo are currently managed similarly to those of cattle, on the assumption that the immune responses are similar. Buffalo are susceptible to many of the diseases affecting cattle, and many of the vaccines and chemotherapeutic regimens developed for use in cattle have proven effective in buffalo (Council, 1981; Tomar and Tripathi, 1987). However, there are also clear differences in the response to some pathogens, emphasizing the need to modify the bovine-based strategies of management to meet the needs unique to buffalo (Council, 1981). To achieve this objective, it will be

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necessary to identify and characterize the immune mechanisms that account for differences in resistance and disease susceptibility observed between cattle and buffalo. Before this can be accomplished, however, the immune system of buffalo needs to be characterized.

Until now, very few studies have been conducted to develop or identify the mAb reagents needed to characterize the immune system of buffalo. This is attributable to the lack of resources and limited number of investigators available to develop mAb reagents needed for research in buffalo. To circumvent these limitations, we have taken advantage of the phylogenetic conservation of the structure and function of orthologous molecules in different species. Investigators that participated in the Third International Workshop on Leukocyte Antigens of Cattle and Sheep screened the mAbs submitted to the workshop for cross reactivity with buffalo LDM (Naessens and Hopkins, 1996; Vilmos et al., 1996). Multiple mAbs were identified, some of which identified epitopes conserved on orthologues in multiple species including buffalo. Of the 159 mAbs examined, 110 reacted with molecules expressed on buffalo leukocytes, indicating it might be possible to use this strategy to identify most of the mAbs needed for studies in buffalo. Sixty five of the mAbs used in the study were submitted by our laboratory. These were included in a subsequent study of over 200 mAbs examined specifically for cross reactivity with LDM in buffalo (Davis et al., 2001). MAbs were identified that were specific for MHC I and MHC II molecules and some specific for orthologues of LDM (numeric name designation for each molecule, cluster of differentiation molecules, CD) characterized in humans. Additional mAbs were identified that recognized epitopes conserved on sIgM, λ light chain, granulocytes, the δ and γ chains of the γδ T cell receptor, the workshop cluster 1 (WC1) molecules, B cells and molecules with upregulated expression on concanavalin A (conA) activated lymphocytes (Davis et al., 2001). Some mAbs were duplicates of mAbs that recognized the same or different epitopes on the same CD molecule. Initial data were obtained on the frequency of leukocyte subsets (Davis et al., 2001). Only a few animals were available for the initial studies, limiting our ability to accurately compare the phenotype and frequency of subsets in animals of different ages. The objectives of the present study were twofold 1) verify cross-reactivity of mAbs identified in the previous studies and validate activity of recently characterized mAbs for cross reactivity with orthologues of buffalo CD molecules and 2) compare and extend information on the composition and frequency of leukocyte subsets in buffalo at different ages.

2. Materials and methods

2.1. Animals

Three young (1.5–2 months of age) and three adult male (2 years of age) Egyptian buffalo (*Bubalus bubalis,* River type - Mediterranean breed), were used in the initial follow up studies of our first reports on buffalo (Davis et al., 2001; Mosaad et al., 2006). All protocols and procedures were approved and guided by Alexandria University under regulations of Egyptian law. The present study was conducted with ten heifers (8–9 months) and fifteen adult buffalo cows (River type - Italian Mediterranean breed) were selected for this study. Adult animals were further divided into three subgroups: five primiparous (3–4 years), five mature (7–8 years, 3–6 lactations) and five old (12–14 years, 6–9 lactations) buffalo cows. The study was carried out at the CREA-PCM farm. The animals involved in this experiment were treated in compliance with the animal testing regulations established under Italian law. The experimental design was carried out according to good veterinary practices under farm conditions. The CREA-PCM is authorized to use farm animals for experimental design (as stated in DM 26/96-4 of Italian Welfare Ministry).

2.2. Monoclonal antibodies used in the study

All monoclonal antibodies (Table 1), except anti-CD335 (clone AKS1, Pierce-Thermo Fisher Scientific, Waltham, MA-USA) were provided by the Washington State University Monoclonal Antibody Center (WSUMAC) (http://vmp.vetmed.wsu.edu/resources/monoclonal-antibody-center, Pullman, WA-USA).

2.3. Sample collection and cell preparation for flow cytometry

Peripheral blood was collected from jugular vein into vacutainer tubes containing Acid Citrate Dextrose solution B (ACD-B) (Becton Dickinson, Plymouth, UK). For general flow cytometry, erythrocytes were lysed with tris-buffered ammonium chloride solution (0.87% w/v, pH 7.3) and the harvested white blood cells were washed in phosphate buffered saline (PBS, pH 7.2). The cells were counted and then re-suspended in PBS (2×10^7 cells/mL) and processed for staining for flow cytometry.

For analysis of molecules upregulated on activated cells, peripheral blood was diluted 1:2 with PBS and then peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Histopaque (density 1.077 g/mL; Sigma-Aldrich, St. Louis, MO-USA). The harvested PBMCs were washed in PBS, counted and cultured in complete RPMI (RPMI medium containing: 10% fetal bovine serum (FBS), 1% glutamine, 0.05 mM β-mercaptoethanol, 50UI/mL penicillin and 10 μg/mL streptomycin) at 2×10^6 cells/mL. The cells were stimulated with 5 μg/mL of concanavalin A-type IV (Sigma-Aldrich, St. Louis, MO-USA) for 5 days. Before flow cytometric (FC) analysis, cells were subjected to density gradient centrifugation to remove dead cells. The harvested viable cells were counted and processed for flow cytometry as described below.

2.4. Labeling of cells for flow cytometry

Single color immunolabeling was used to show which of the mAbs listed in Table 1 reacted with buffalo leukocytes and yielded patterns of labeling consistent with the patterns of labeling obtained from cattle using SSC vs fluorescence (Davis et al., 2001). Staining of cells for flow cytometry was done as previously described (Elnaggar et al., 2015; Park et al., 2016). In brief, the cells were incubated with mAb (0.75μg/10^6 cells) for 15 min at 4 °C in the dark. Cells were washed three times (400 g for 3 min) then incubated under the same conditions with 100 μL of a 1:200 dilution of fluorescein (FITC)-conjugated goat anti-mouse IgG/A/M (AbD Serotec, Kidlington, UK). The cells were washed twice and fixed for 30 min with 2% (v/v) PBS buffered formaldehyde and kept in the refrigerator until examined.

Multi-color immunolabeling was performed using combinations of mAbs (Tables 2 and 3) as above using isotype specific secondary antibodies at optimal dilutions: 1:1000 Alexa flour 647 anti-mouse IgG1, 1:800 PE-Cy7 anti-mouse IgG2a, 1:1500 PE anti-mouse IgG2b, 1:400 PE anti-mouse IgM, 1:1600 Alexa flour 488 anti-mouse IgG3.

2.5. Flow cytometry and data analysis

Cells were collected on a FC500 flow cytometer (Beckman Coulter, Miami, FL-USA) and data was analyzed using FCS Express (DeNovo, Glendale, CA-USA) and Kaluza software (Beckman Coulter, Miami, FL-USA). Selective electronic gates were used to isolate cell subsets for analysis. Following single mAb