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Flow Cytometric Characterization of Lymphocyte Subpopulations in the Korean Bovine

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Abstract: Characterization of immune cell subpopulations in the bovine was performed using a direct immunofluorescence technique adaptable for routine and repeated monitoring. This whole blood procedure is faster and requires less volume than conventional density gradient isolation methods. Low inter-and intra-animal variations were seen in hematology parameters and in CD4, CD8 and CD2 lymphocyte subtypes. CD4 values were 30% of lymphocytes in male and 32% in females. Thriteen percent were CD8 in males and 13% in females. CD4: CD8 ratios were approximately two in both sexes. Fiffty three percent were CD2 in males and 54% in femals. The mean RBC counts of peripheral blood were $7.20 \times 10^6/mm^3$ for male cattle and $6.36 \times 10^6/mm^3$ for females. The mean WBC counts were $8.09 \times 10^3/mm^3$ for males and $7.09 \times 10^3/mm^3$ for females. The percent of lymphocytes(63-65%) was higher than the percent of neutrophils(17-18%), the percent of eosinophils(11-15%), te percent of monocytes(4-5%), and percent of basophiles(1%).

Key Words: leukocyte subopulation, peripheral blood, monoclonal antibody, flow cytometry.

INTRODUCTION

The successful application of monoclonal antibody technology has led to the identification of fuctionally distinct subsets of morphologically similar lymphocyte cell populations in many mammalian species. Studies of humans, rats, mice, pigs, sheep and monkies suggest that molecules specific to fuctional subsets of lymphocytes are highly conserved during evolution[2,4,10,14]. Bovine also is on important animal model for the study of zoonoses and transmitted diseases. Bovine, however, has not been widely used to examine immune function during the preclinical development of biochemical entities. Reasons for this include a limited normal base, lack of accepted or validated methologies, unknown prior care or medical history, cost and low numbers of animals used per treatment group. While bovine possess an immune system anatomically[3a, 3b] and functionally[3a, 3b] similar to humans, little information is available regarding lymphocyte subpopulation characterization and immune function in the bovine. The species is commonly used in pharmaceutical evaluations of efficacy

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and toxicity and are available in reasonable numbers. This study was initiated to establish normal ranges for CD2, CD4 and CD8 lymphocytes, together with ancillary hematology parameters, using a labor efficient procedure. It was deemed important that these techniques utilize small quantities of peripheral blood. The work was conducted using peripheral blood without density gradient separation of mononuclear cells[1,5,6,7]. This technique can be used to obtain data on lymphoid cell population from studies monitoring routine clinical immunology and hematology profiles, without additional handling of the animals or the need for separate studies.

MATERIALS AND METHODS

Donor cattle

The cattle were 12- to 20-week-old Holstein steers, acquired 1-2 days of age and housed individually in concreate stalls of Yonsei stock farm. The animals were maintained on a hay diet supplemented with grain. Water was supplied *ad libithum*. Twelve female and ten male cattle were sampled for determining hematology and lymphocyte subpopulation parameters.

Hematological exam

Leukocyte differntial counts were performed on smear made from the EDTA blood sample. Wright stained blood smears were evaluated using a mannual method programmed for the differential analysis of bovine cells. Total white blood cell(WBC) and red blood cell (RBC) counts were determined on a Coulter Model S-Plus IV(Coulter Electronics).

Lymphocyte characterization

Whole blood was collected from the jugular vein in Dulbecco's phosphate buffered saline (D-PBS) plus sodium citrate at a ratio of 9 parts whole blood to 1 part D-PBS. This was mixed 1:1 with Hank's balanced salt solution (HBSS) without calcium and magnesium with 10mM EDTA. Mixtures containing bovine blood overlayed on Ficoll-Paque at a 20 ml of blood-HBSS to 9 ml of Ficoll-Hypaque in a 50 ml polystyrene centrifuge tube. The resulting gradient was centrifuged at $700 \times g$ for 45 minutes. Bovine peripheral blood lymphocytes (PBL) were then collected, washed three times in HBSS, and suspended in RPMI-1640 supplemented with 5×10^{-5} M2-mercaptoethanol, 25 mM Hepes. 100 U ml^{-1} penicillin, 100 ug ml^{-1} streptomycin and 5% fetal bovine serum (FBS).

Monoclonal antibodies specific for bovine lymphocytes surface markers were used as ascites fluids, with the exception of Control-R(C-R) which were used as culture supernatants. C-R is an IgG2a mAb that recognizes a monomorphic determinant on class 1 antigens. three of these differentiation antigens, CD2, CD4 and CD8, are detectable on almost all mature peripheral human T lymphocytes. All entities appear to function in the activation of T cells.

Cell surface phenotype was assessed by indirect immunofluorescence. Briefly, $1\text{-}2 \times 10^{6}$ cloned cells were washed with fluorescence buffer(FB) consisting of PBS containing 0.01% sodium azide and 0.1% bovine serum albumin (BSA). The cells were added to 12×75 mm tubes, incubated on ice for 30 min with the first stage antibody, then washed twice with FB. FITC-conjugated goat anti-mouse IgG 25 ul (Cooper Biomedical, Inc. Malvern, PA) was added to the cells prior to incubation for an additional 30 min on ice. After two more washes, an aliquot was taken to determine cell viability by Trypan Blue exclusion. Finally, 0.4 ml of cold fluorescence fixative(PBS, 0.01% sodium azide, and 1% formaldehyde) was added. The number of cells stained was determined using an Epics Profile Analyzer(Coulter Corpoation, Hialeah, FL).

Statistical analysis

The data were expressed as a percent of po-

Table 1. Hematologic parameters of bovine in Korea.

Parameter	Male	Female
RBC($\times 10^6$/mm^3)	7.20±0.09	6.35±0.08
WBC($\times 10^6$/mm^3)	8.09±0.5	7.09±0.3
Neutphils(%)	17±2	18±1
Lymphocytes(%)	63±1	65±1
Monocytes(%)	4±0	5±0
Eosinophils(%)	15±1	11±1
Basophils(%)	1±0	1±0
Platelet($\times 10^5$/mm^3)	3±0	3±1

Table 2. Lymphocyte subpopulation characteristics of bovine

Males(%)		Females(%)
CD2	53.2	54.3
	(57.4-45.5)	(57.9-47.7)
CD4	30.3	32.4
	(35.3-28.5)	(37.3-30.9)
CD8	13.2	12.8
	(16.5-10.6)	(15.1-8.9)

sitive cells and the absolute number of lymphocytes per liter of blood. Comparisions of the male and female means for the entire colony were performed using the Student's t-test. For the male and female subsets examined at three time points, linear trend effects were evaluated using a one-factor at three time points, linear trend effects were evaluated using a one-factor analysis of variance on rank-transformed data(non-parametric) for each sex. A 5% significance level was used to determine statistical significance. Variability attributable to sampling and staining conditions was calculated as the mean standard deviation of each set of replicates and expressed as a percentage of the mean value for each cell surface marker.

RESULTS

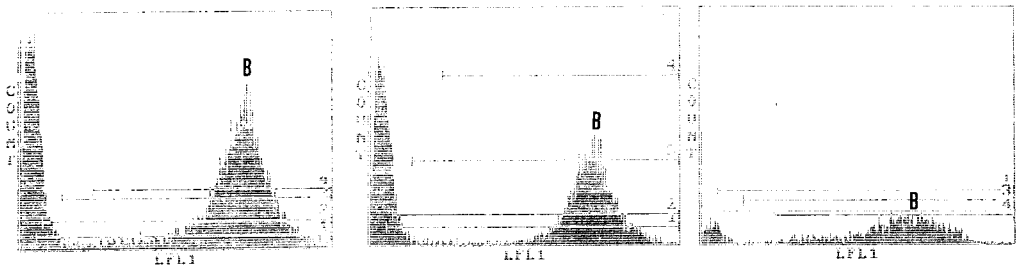
Hematological examination

Hematologic parameters for the full colony are shown in Table 1. No significant differences were seen between males and females in RBC, WBC, PLt or leucocyte differential counts. A 100-cell differential count. A 100-cell differential count was performed on each sample. The mean percent of lymphoytes was 63 for males and 65 for females. The mean percent of neutrophils was 17 for males and 18 for females. The mean percent of eosinophils was 15 for males and 11 for females. The mean percent of monocytes was 4 for males and 5 for females. The mean percent of basophils was 1 for both males and females.

Lymphocyte characterization

Males and females had similar percentages and absolute numbers of CD2, CD4 and CD8 lymphocytes(Table 2). The ratio of CD4, CD8/CD2 positive cells was also comparable in both sexes. These three lymphocyte surface markers accounted for more than 95% of the peripheral blood lymphocytes. In the animals sampled three times, no significant trends of differences were found between the collection dates, The total of the cells identified by the three markers were 92-100% in males, 87-100% in females. No significant differences were seen in lymphoid parameters between males and females in the sample.

When samples from males and female bo-



(A) (B) (C)

Fig. 1. Comparative distribution of bovine T lymphocyte differentiation markers as revealed by Epics profile analyzer. Bovine peripheral blood reacted with monoclonal antibodies (A) CD2, (B) CD4 and (c) CD8. B: Peaked for bovine lymphocyte stained.

vines were analyzed in triplicate, good agreement was seen for all three lymphocytes markers(data not shown). The intra-sample variability attributable to sampling and the staining procedure was 4% of the mean for CD2 cells. The values for CD4 and CD8 were 5.2% and 5.5%, respectively.

The histograms in Fig. 1 show the staining patterns of bovine lymphocytes. They give well defined positive and negative peaks for CD2, CD4 and CD8.

DISCUSSION

The lymphocyte subpopulation examined in the current study were selected because of their clinical relevance and importance. The use of antibodies directed against the CD2, CD4 and CD8 cell surface markers allows differentiation of human T-lymphocytes into regulatory/functional subpopualtions[6,7,9]. We found the bovine to exhibit good staining intensities and relatively low inter-and intra-animal variabilities in numbers of CD2, CD4 and CD8 peripheral blood lymphocytes when labeled with commercially available human reagents. All the T cell clones tested expressed the CD2, CD4 and CD8 of peripheral blood lymphocytes positive for this marker. This conclusion is based on the molecular and peripheral blood lymphocyte distributions of the target molecules and their association with certain typical functional characteristics of T lymphocyte subpopualtions in concordance with the numbering system applied to the human clusters of differentation.

According to recently studies many of the leukocyte differentiation molecules first defined in rodents and humans are highly conserved with respect to their antigenic composition and expression on leukocytes. This has permitted the use of FC to compare the patterns of expression of MoAb-defined molecules prior to hematological and functional analysis. It has also allowed MoAbs from conserved determinants to be used to establish that the same molecules have indeed been identified in different species. MoAbs to MHC class I and class II molecules, BoCD2, BoCD4 and BoCD8 were established in previous studies by these methods[3a,3b]. From the FC and immunohistochemical value obtained references the list can now be extended to include the orthologues of CD1, CD2, CD4, CD 5, CD6, CD8, CD11a, CD11b, CD11c, CD44, CD45, CD58 and a series of new differentiation molecules, some of which have as of yet, no identified CD equivalents[7,9,11,13,15]. Further investigation will undoubtedly reveal that some of these molecules are orthologues of known CD molecules and others orthologues of undefined molecules. The continued use of the comparative approach to the study of leukocyte differentiation molecules should greatly facilitate definition of the immune system in vertebrates.

The finding that the composition of the lymphoid system differs in some species is of exceptional intrest since the noted differences provide unique opportunities to elucidate the biological significance of such variations as well as opportunities to examine the regulatory and effector activity of populations of cells that are generally in low concentration in humans and rodents. For example, a relatively large population of $CD^+/CD8^+$ T-cells are found in the peripheral blood of pig[8]. The function of these cells in MHC restricted and unrestricted immune responses is unknown. In addition, the concentration of these cells in peripheral blood is sufficiently high to permit analysis of their regulatory and effector activites.

In young animals, the T-cell population may comprise 40% to 80% of the circulating lymphocytes[2,5,6,7,12]. The abundance of these cells in young animals affords an opportunity to examine the functional activity of each population. It also affords an opportunity to examine the functional activity of each population. It also affords an opportunity to detail the phylogenetic and phenotypic relation of these cells to those under investigation in humans and rodents. In addition, the MoAbs that define the lineage-specific molecules provide the means for isolation and characterization of the genes that encode the molecules in ruminants and other species.

The results this study, hematologic parameters(RBC, WBC, PLt, leukocyte differential counts) of peripheral blood, are very similar with other studies. There was no significant difference between male and females cattles (Table 1). In this study, the percent of eosinophils(11-15%) was higher than in other animals and human lymphocytes were the principal leukocyte in the peripheral blood.

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= 국문초록 =

한우 혈액내 백혈구아군의 특성 및 말초혈액상

연세대학교 보건과학대학 임상병리학과 및 문리대학 생물자원공학과*

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소는 인간에게 고기와 우유를 제공하는 영양자원으로서 뿐만아니라 노동력을 제공하는 중요한 가축 동물 중의 하나이다. 또한 사람의 질병을 규명하고 예방 및 치료의 실험적 도구로서도 많이 이용되고 있으나, 대동물이라는 점과 경제적인 이유로서 일반적인 응용과 실험적 적용에 어려움이 되어왔다. 저자들은 이와같은 사정을 감안하여 실험적 기초자료를 얻기 위하여, 한우를 대상으로 말초혈액상과 특히 림프아군의 특성을 알아본 결과 다음과 같이 일부 결과를 얻었다. 즉, 한우 22마리(암컷 12마리, 숫컷 10마리)의 말초혈액을 채혈하여 림프구 표면항원의 특성을 단클론항체와 반응시키고 flow cytometry로 측정한 결과 CD2는 숫컷에서 53%, 암컷에서 54%의 반응을 보였고, CD4는 숫컷에서 30% 암컷에서 32%의 반응을 보였으며, CD8에서는 숫컷에서 13%, 암컷에서 13%의 반응을 보였다. 그리고 말초혈액상은 RBC가 숫컷 $7.20\times10^{6}mm^{3}$, 암컷 $6.35\times10^{6}mm^{3}$이었고 WBC는 숫컷 $8.09\times10^{3}mm^{3}$, 암컷 $7.09\times10^{3}mm^{3}$이었으며 leukocyte differential counts 에서는 lymphocytes, Neutrophils, Eosinophils의 순으로 높은 성적을 보였다.

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