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Distribution of T and B lymphocytes in peripheral blood and lymphoid tissues of bovine

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소의 순환혈액 및 림프조직내 T 및 B 림프구 분포

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초록 : 소의 순환혈액 및 림프조직내의 T 및 B 림프구의 분포를 파악하고자 소흉선 세포를 토끼에 과면역시켜 얻은 항혈청을 소의 적혈구, 간장분말 및 골수세포에 순차적으로 흡착시킨 항 흉선 세포혈청(RABTS)과 성숙 T 림프구에만 특이성을 가진 단클론항체 BLT_1 및 B 림프구와 소수의 대식세포에 특이성을 가진 단클론항체 $_6E_{12}$를 1차 항체로 이용하여 ABPC법으로 T 및 B 림프구를 동정한 바 다음과 같은 결과를 얻었다.

RABTS 및 BLT_1으로 동정한 순환혈액내 단핵세포(PB-MNCs) 중 T림프구의 비율은 각각 70.9±5.5% 및 59.0±8.7% 이었으며, PB-MNCs중 nylon wool 비부착세포를 BLT_1 및 $_6E_{12}$로 동정한 T 및 B 림프구의 비율은 각각 91.6±1.0%, 9.6±0.8%이었다. 또한 BLT_1 및 $_6E_{12}$로 동정한 서혜 림프절, 장간막 림프절, 비장 및 흉선의 T/B림프구의 비율은 각각 56.4±6.2/45.3±7.4%, 55.6±7.7/42.3±5.8%, 48.6±5.1/48.5±6.2% 및 23.0±4.8/5.6±2.1%이었으며, RABTS로 동정한 이들 림프조직내의 T림프구의 비율은 각각 76.3±3.4%, 74.2±8.2%, 73.6±5.5% 및 95.6±2.8%이었다.

이상의 결과로 미루어 PB-MNCs내의 T림프구 분리는 nylon wool column법이 보다 효과적임을 알 수 있었다.

Key words: monoclonal antibodies, avidin-biotin peroxidase stain, mononuclear cells, peripheral blood, lymphoid tissues, Korean cattle.

Introduction

In order to evaluate activity of the host immune system, an identification of immune-regulatory cells and their activity have been intensively studied.[1~5]

In the bovine system there has been difficulty in finding immunological characteristics because of few creditable methods in immunological research. It has become necessary, then, to find methods of separating immune-regulatory cells and their respective functions.[3~7] Consequently, many methods have been developed to measure the activity of immune-regulatory cells and their functions under normal and abnormal conditions.[3~5,8,9]

Several methods using immunoperoxidase exist including;[10,11] hybrid method,[12] direct and indirect peroxidase antibody method,[10] enzyme bridge(EB) method[13] and peroxidase antiperoxidase(PAP) method.[14] Among these, EB and PAP use unconjugated antibody, which is unique when compared to immunofluorescence and most other immunoenzymatic techniques. This may be key to the extremely high

sensitivity and specificity of the methods, since chemical conjugation of an enzyme to an antibody frequently results in decreased immunochemical reactivity of the antibody as well as the catalytic activity of the enzyme. Therefore, these methods permit the specific demonstration of the cell and tissue antigens in a variety of the fixed tissues.

The purpose of the present report was to study the normal distribution of T and B lymphocytes in blood and other lymphoid tissues of bovine, using specific membrane markers of T and B lymphocytes by the method of avidin-biotin peroxidase complex (ABPC), a type of EB.

Materials and Methods

Blood and Tissues: Bovine blood was obtained from 1~3 years old Korean cattle at Chonbuk Animal-Breeding-Center, and lymphoid tissues of bovine such as inguinal lymph nodes, mesenteric lymph nodes, spleen and thymus were obtained from a local slaughter house(Palbok-dong, Chonju).

Mononuclear cells(MNCs):[15] Peripheral blood mononuclear cells(PB-MNCs): Bovine blood was collected in heparinized(20IU/ml) syringe from clinically healthy Korean cattle by jugular veinfuncture and was diluted 1 : 1 with phosphate buffered solution(PBS, pH 7.2). Buffy coat cells were separated by centrifugation at 400g for 20min and layered over the same volume of Ficoll-Hypaque solution (F-H, Sigma, d=1.083). After centrifugation at 400g for 40min, PB-MNCs at the F-H interface were collected and rinsed three times with PBS at 400g for 10min. Then, these PB-MNCs were resuspended in RPMI-1640 containing 10% fetal calf serum (10% FCS-RPMI 1640; medium).

Lymphoid tissues-MNCs: The lymph nodes, spleen and thymus were dissociated in PBS and passed through wire-mesh(#100) to obtain a free cell suspension. These MNCs were washed and resuspended as described above.

Nylon wool column: Purified T lymphocytes in PB-MNCs were obtained by nylon wool columns.[9] Briefly, nylon wool columns were prepared by loosely packing 10ml plastic syringes with 0.6g of scrubbed nylon fibers(Cellular Products Inc. NY, U.S.A.) which had been teased apart. Autoclaved columns were preincubated with medium at 37°C for 1hr. The columns were then washed with 5ml warm medium and 0.5ml of the cell suspension(2×10^7 cells/ml) was run into column, followed by 0.5ml of warm medium. The columns were incubated horizontally at 37°C for 1hr. The nylon wool nonadherent cells were collected by the dropwise addition of 30ml of warm medium, centrifuged and resuspended to 5ml medium.

Polyclonal rabbit anti-bovine thymocytes serum (RABTS):[1,16] The RABTS was prepared two rabbits given (a) 2 intravenous injections of 1×10^9 washed calf thymocytes in phosphate buffered solution (PBS) and then (b) multiple-site intramuscular injections of 5ml of thymus homogenate Freund's incomplete adjuvant(1 : 1, v/v of 50% tissue homogenate and adjuvant). Blood was harvested from the rabbits 11 days after the last injection. Heat-inactivated serum was adsorbed twice with equal volumes of packed bovine RBC, once with an equal volume of lyophilized normal bovine serum at 37°C for 30min, and once each with calf bone marrow cells(1 part of marrow cells and 2 parts of anti-serum) at 37°C for 30 min and at 4°C overnight. At this time, the adsorbed serum was negative for RBC and bone marrow cells, but positive for thymocytes(at 1 : 64), as determined by agglutination tests. The adsorbed anti-serum was then clarified with a Millipore filter, and antibodies were precipitated by ammonium sulfate, redissolved in PBS and titrated by the leukoagglutination test. The subagglutination dose was assayed for specificity by indirect fluorescent antibody (IFA) techniques, using fluorescein isothiocyanate-conjugated (FITC)-goat anti-rabbit IgG(Sigma). It reacted with more than 95% of the thymocytes and less than 5% of the F-H separated bone marrow cells. Preadsorption with thymocytes abolished the activity. In the absence of RABTS, fluorescent cells were not seen among thymocytes and bone marrow cells.

Primary antibodies: Monoclonal antibodies(MAbs), BLT_1 and $_6E_{12}$ were kindly provided from Dr. Thomas Yang, University of Connecticut, and RABTS were used in avidin-biotin peroxidase complex

(ABPC; Vector Laboratories; Burlingame, CA) staining as primary antibodies. Among these primary antibodies, BLT_1 and $_6E_{12}$ have specificity for bovine mature T cells and bovine B cells(a small number of other cells also, because of contaminating Fc receptor-positive MNCs), respectively.

Identification of T and B lymphocytes: T and B lymphocytes in MNCs were identified by the ABPC staining method as described previously.[17]

Briefly, 1ml of cell suspension(1×10^7 cells/ml) of each MNCs was treated with 0.3% H_2O_2 in methanol 30min and washed, followed by 3% normal goat (horse) serum for 20 min to block the endogenous peroxidase activity and to reduce the nonspecific background staining, and was washed once with Tris-buffered saline(TBS, pH 7.6). Then, the cells kept overnight at 4°C with primary antibodies diluted appropriately, washed and treated with biotinylated-goat anti-rabbit IgG (H+L; Vector) for RABTS or biotinylated-horse anti-mouse IgG(H+L; Vector) for BLT_1 and $_6E_{12}$ diluted 1/200 for 1 hr and washed three times with TBS. The cells were treated with ABPC diluted 1/200 for 1hr and washed three times with TBS. The final reaction was achieved by incubation the cell suspensions with substrate mixture (3,3-diaminobenzidine, DAB; Kirkegaard & Perry Laboratories Inc.; Gaithersburg, MD) for 5min and these reactants were washed once with TBS.

Cell suspensions were examined under microscope and at least 200 cells were counted. Cells completely encircled by red brown ring of staining were counted as positive. Finally, immunoperoxidase positive cells were calculated as follows;

$$\%\text{ of ABPC positive cells} = \frac{\text{Positive number of ABPC}}{\text{Cells counted}} \times 100$$

Results

BLT_1, $_6E_{12}$ and RABTS positive cells in PB-MNCs: As shown in Table 1, BLT_1 reacted with 59.0±8.7%, $_6E_{12}$ with 23.0±8.7%, and RABTS with 70.9±5.5% of PB-MNC. Further studies showed that over 91% of nylon wool nonadherent cells were BLT_1 positive.

RABTS and BLT_1 positive cells in lymphoid tissues-MNCs: As shown in Table 2, RABTS reacted with 76.3±3.4%, 74.2±8.2% and 73.9±5.5% of the MNCs from inguinal lymph node, mesenteric lymph node and spleen, respectively. In contrast, RABTS reacted with 95.6±2.8% of thymocytes.

BLT_1 reacted with 56.4±6.2%, 55.6±7.7%, 48.6±5.1% and 23.0±4.8% of the MNCs from inguinal lymph node, mesenteric lymph node, spleen and thymus, respectively.

$_6E_{12}$ positive cells in lymphoid tissues-MNCs: As shown in Table 2, $_6E_{12}$ reacted with 45.3±7.4%, 42.3±5.8%, 48.5±6.2% and 5.6±2.1% of the MNCs from inguinal lymph node, mesenteric lymph node, spleen and thymus, respectively. Because $_6E_{12}$ has specificity for B lymphocytes and a few other cells, these data may be slightly higher than the

Table 1. Cell type distribution before and after nylon wool column in bovine PB-MNCs

Cell types	% of positive cells in PB-MNCs	
	Before passage*	After eluted
B-cells	23.0±8.7	9.6±0.8
T-cells	59.0±8.7	91.6±1.0

Separation by F-H gradient was shown to be effective for separating mononuclear cell fraction from peripheral whole blood. Further enrichment of T cells by passage through nylon wool column yield the suspension of approximately 92.0% purity. T and B cells were identified by BLT_1 and $_6E_{12}$ using ABPC method. Values are expressed as Mean±S.D. from 5 animals.

* Percentage of PB-MNC with RABTS was 70.9±5.5%.

Table 2. Distribution of T and B lymphocytes in various lymphoid tissues-MNCs

MNCs from	% of positive cells		
	RABTS	BLT_1	$_6E_{12}$
Inguinal lymph node	76.3±3.4	56.4±6.2	45.3±7.4
Mesenteric lymph node	74.2±8.2	55.6±7.7	42.3±5.8
Spleen	73.9±5.5	48.6±5.1	48.5±6.2
Thymus	95.6±2.8	23.0±4.8	5.6±2.1

Percent cells estimated by ABPC stain.
Values of RABTS, BLT_1 and $_6E_{12}$ are expressed as Mean±S.D. from 10 animals.

true value of B lymphocytes.

Discussion

Identification of lymphocyte subpopulations is of great importance in understanding their functions in health and disease conditions. In cattle, it has been hampered by the lack of reliable techniques to identify the T and B lymphocytes. Therefore, a method for the measurement of bovine T and B lymphocytes has been desired. We adapted ABPC staining method for these purpose using RABTS, BLT_1 and $_6E_{12}$ as primary antibodies.

In the present experiment, we prepared a T-cell antigen specific anti-thymocyte serum(RABTS), enabling us to identify more than 95% of the thymocytes and more than 70% of the PB-MNCs as T lymphocytes.

Recently, similar studies have been reported. In those reports, the anti-bovine thymocytes serum was developed by inoculating goat (GABTA)[1] or rabbit (ATS)[16] with calf thymocytes. Concerning specificity, it was noted that both GABTA and ATS were nonreactive with bone marrow cells but reactive with thymocytes. Yang[1] said that there were 72.9±5.7% T lymphocytes using GABTA by indirect fluorescent antibody(IFA) and 26.9±4.4% B lymphocytes by direct FA in PB-MNCs. Nakanishi *et al.*[16] said that there were 80.4% T lymphocytes and 9.6% B lymphocytes in nylon wool nonadherent cells of PB-MNCs, 59.4±6.8% T lymphocytes and 28.2±5.6% B lymphocytes in lymph node, and 43.4±4.8% T lymphocytes and 48.7±4.7% B lymphocytes in spleen using ATS by IFA for T lymphocytes and direct FA for B lymphocytes. On the other hand, Rabinovsky and Yang[2] said that there were 92.3±2.7% T lymphocytes and 2.2±0.5% B lymphocytes in nylon wool nonadherent cells of PB-MNCs, 61.1±2.7% T lymphocytes and 37.6±3.0% B lymphocytes in prescapular lymph node, 67.2±4.9% T lymphocytes and 24.5±2.1% B lymphocytes in supramammary lymph node, 28.8±2.7% T lymphocytes and 22.5±2.5% B lymphocytes in spleen, and 25.3±2.8% mature T lymphocytes and 0.4±0.3% B lymphocytes in thymus using BLT_1 with IFA for T lymphocytes and direct FA for B lymphocytes. These values similar to those obtained by the authors, although T lymphocyte percentage of lymph node was slightly higher than ours and T/B cell percentage of spleen was different from one another. However there have been few investigations of the spleen and lymph nodes, then, desired more studies about distribution of T and B lymphocytes in these lymphoid tissues.

Apparently, the rates of GABTA, ATS or RABTS positive cells were higher than that of BLT_1, indicating that the T cell epitopes identified by polyclonal antibodies and by monoclonal antibody were different and also there was difference of specificity between polyclonal antibodies and monoclonal antibody.

Summary

This study was undertaken to identify the distribution of T and B lymphocytes in bovine peripheral blood and various lymphoid tissues by the method of ABPC using RABTS, BLT_1 and $_6E_{12}$ as primary antibodies.

RABTS, BLT_1 and $_6E_{12}$ positive cells in PB-MNCs were 70.9±5.5%, 59.0±8.7% and 23.0±8.7%, respectively. BLT_1 and $_6E_{12}$ positive cells in nylon wool nonadherent cells of PB-MNCs were 91.6±1.0 % and 9.6±0.8%, respectively.

In the lymphoid tissues such as inguinal lymph node, mesenteric lymph node, spleen and thymus, positive cells of RABTS were 76.3±3.4%, 74.2±8.2%, 73.9±5.5% and 95.6±2.8%, those of BLT_1 were 56.4±6.2%, 55.6±7.7%, 48.6±5.1% and 23.0±4.8% and those of $_6E_{12}$ were 45.3±7.4%, 42.3±5.8%, 48.5±6.2% and 5.6±2.1%, in order.

These results are indicating that nylon wool column method is effective for separation of bovine ocytes.

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