正常牛の末梢血,リンパ節,脾臓におけるT,Bリンパ球の測定

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Identification of Bovine T and B Lymphocytes in Normal Peripheral Blood, Lymph Nodes and Spleen

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ABSTRACT. Anti-bovine thymocyte serum (ATS) was prepared by immunizing rabbits with calf thymocyte, adsorbed by normal bovine red blood cells, liver powder and bone marrow cells, and applied to identify bovine T lymphocytes by indirect immunofluorescent antibody technique (FA). The frequency of T and B lymphocytes in the PBL, spleen and lymph nodes of 21 clinically normal cattle were investigated by indirect FA using ATS and by direct FA using FITC rabbit anti-bovine serum, respectively. Distributions of T and B lymphocytes were 66.4±6.4% and 25.4±3.9% in the PBL, 43.4±4.8% and 48.7±4.7% in the spleen and 59.4±6.8% and 28.2±5.6% respectively in the lymph nodes.

Two major classes of lymphocytes, T and B cells, have been found in animals. Bovine B-cells can be identified by erythrocyte-antibody-complement (EAC) rosettes [12], erythrocyte antibody [EA] rosettes [11] and surface immunoglobuline (SIg) immunofluorescence assay [4, 6, 11, 16]. Bovine T cells have been identified by erythrocyte (E) rosette [1, 10, 11, 12, 14] formation. Bovine T cells have been identified by E rosette formation. This method tends to be variable and has poor detection sensitivity, and therefore, a more effective method of detecting T lymphocytes is desired. To solve this problem, the authors prepared anti-bovine thymocyte serum (ATS) and obtained results which are reported here.

MATERIALS AND METHODS

Animals: The PBL, spleen and lymph nodes from 21 clinically normal cattle (2 to 8 years old, Japanese Shorthorn; JS, Holstein-Friesian; HF, Japanese Black; JB) were obtained from an abattoir (Aomori prefecture, Japan). These cattle were negative for both bovine leukosis virus (BLV) and BLV antibody as determined by lymphocyte culture and double gel immunodiffusion respectively [5, 7, 8].

Anti-bovine thymocyte serum (ATS): The thymus of two clinically normal calves of the JS breed was teased to provide single cells which were washed three times. Contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride buffer [3], was washed three times and adjusted to 5×10^9 cells per m*l* with phosphate buffered saline (PBS). Five milliliters of this cell suspension was mixed with the same volume of complete adjuvant and the mixture was inoculated into two rabbits at several sites intramuscularly and subcutaneously. After 12 days, additional immunization was performed by the same method. The animals were sacrificed 13 days after the final inoculation and serum was obtained. After heat inactivation, the rabbit sera were absorbed three times with

three volume of packed red blood cells, twice with 0.1 g of bovine liver powder per m*l* and 11 times with 1×10^8 bovine bone marrow cells per m*l*, and the resulting serum was used as the ATS. The ATS of the two rabbits was divided into small amounts separately and kept at −80°C until use.

Immunofluorescent (FA) technique: A direct FA technique was used to identify B lymphocytes and an indirect FA method was used to identify T lymphocytes. One m*l* of lymphocytes (2×10^6 cells) was centrifuged at 350 g for 5 minutes and the cell pellet was suspended in 0.1 m*l* of ATS or FITC rabbit anti-bovine IgG serum (1:4 dilution, 4 units, Medical and biological Lab., Ltd. Japan) and reacted at 4°C for 30 minutes. Cells were then washed 3 times with PBS. To identify T lymphocytes, the packed cells were suspended in 0.1 m*l* of FITC goat anti-rabbit IgG serum (1:20 dilution, Medical and Biological Lab., Ltd. Japan) and reacted at 4°C for 30 minutes. Cells were then washed three times with PBS. These washed cell pellets were resuspended in 50% glycerol in PBS. The number of positive cells was determined by counting 200 cells with a fluorescent microscope. All of the antisera used in the test were diluted with PBS containing 0.1% sodium azide. The cells reacted with these antisera were washed with PBS containing 0.5% sodium azide.

Lymphocyte preparation: Thymus, spleen and prescapular lymph node were collected in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and teased in the same medium. The cell suspension were filtered through stainless mesh to obtain a single cell suspension. Whole blood was collected with EDTA, as an anticoagulant, and centrifuged at 900 g for 30 minutes. The cells in the buffy coat were removed, diluted with MEM containing 10% FCS. The cell suspensions obtained from thymus, spleen, lymph node and blood were placed on the Ficoll-Conray mixture (9% Ficoll 400, 96 volumes, Pharmacia Fine Chemical AB, Sweden, and 33.4% Conray, 40 volumes, Daiichi Seiyaku Co. Ltd. Japan), and centrifuged at 400 g for 30 minutes [2]. Lymphocytes forming a layer were collected and washed three times with PBS, and their viability was tested by the trypan blue exclusion method. Cell preparation with a viability of more than 80 per cent of thymus, spleen and lymph node cells, and more than 97 per cent of PBL were used.

Procedure for E, EA and EAC rosettes: E rosette technique was used to identify T-lymphocytes. EA and EAC rosettes technique were used to identify B-lymphocytes. These procedure were all performed according to the methods of previous workers. For the E rosettes, sheep red blood cells (SRBC) were treated with 6% W/V dextran (Sigma, Mol. Wt. 176800 St. Louis) 0.9% NaCl and 0.5% SRBC were used [14]. For the EA rosette, chick red blood cell (CRBC) were sensitized with anti-CRBC-IgG (1:80 dilution, Japan Immunoresearch Lab., Japan) [11]. For the EAC rosettes, trypsin treated SRBC were used [15]. After these SRBC were sensitized with anti-SRBC IgM (1:320 dilution, Japan Immunoresearch Lab., Japan) they were reacted with mouse serum as complement and then used [3]. PBL from normal cattle were testd by E and EAC rosetting techniques. Each tested sample was placed on Ficoll-Conray mixture and separated into rosetting and non-rosetting cells. The cells fractionated by the respective methods were dispersed and the lymphocytes were stained by ATS and SIg.

Treatment of lymphocyte with ATS and com-

plement and lymphocyte stimulation test: Four milliliters of ATS (1:4 dilution), 4 m*l* of PBL suspension (2×10^7 cells per m*l*) and 4 m*l* of rabbit serum as complement source were mixed and kept at 37°C for 45 minutes. Part of the residual lymphocytes from this cytotoxicity test were stained with ATS and SIg. Residual lymphocytes were also used for the mitogen stimulation test. The method for lymphocyte stimulation test previously described was modified [9]. Phytohemagglutinin (PHA-P, Difco Lab., Detroit, Mich), Concanavaline-A 2x cryst (Con-A, Miles Lab., Inc, Elkhart, Ind), and lipopolysaccharide (LPS, Difco Lab., Detroit, Mich) were used as mitogen. PBL were suspended in RPMI 1640 medium containig 20% FCS and antibiotics to a cell concentration of 2×10^6 per m*l*. Mitogen (20 μl) was added to 0.2 m*l* of lymphocyte suspension to obtain a final concentration of 10 μl of PHA per m*l*, 12.5 μg of Con-A per m*l*, or 18.5 μg of Con-A per m*l*. The cultures were incubated for 54 hours at 37°C in a CO_2 incubator and pulsed with 0.2 μCi of [^{3}H] thymidine 18 hours prior to termination, and the uptake of [^{3}H] thymidine was determined. The stimulation index was expressed as the mean count per minute of 3 replicates of mitogen-stimulated cultures divided by the mean count per minutes of 3 replicates of unstimulated cultures.

Separation of Nylon-adherent and non-adherent cells: Lymphocytes were separated on nylon wool columns into adherent and non-adherent populations by a procedure described by previous workers [10]. Columns were prepared in 12 m*l* syringes packed with 0.6 g nylon wool (Fenwal Lab., Leuko-Pak Leukocyte Filter, 4C-24-01, Div. of Travenol Lab., Inc., Deerfield, Illiois). PBL (15×10^7 cells per m*l*) were applied to each column and incubated. The columns were slowly washed and the cells eluted in the first 20 m*l* were pooled and referred to as non-adherent cells. The adherent cells were harvested by washing the nylon wools in PBS containing 5% FCS.

Results

Determination of the specificity of ATS:

For determination of the antibody titer of ATS, a two-fold serial dilution of ATS was reacted with PBL. The PBL was then suspended with FITC rabbit anti-bovine IgG serum and observed by a fluorescent microscope. From these results, a 32-fold dilution of ATS was taken as one unit of antibody titer. In the subsequent experiment, eight dilutions (four units) were used in all cases except for the treatment of lymphocytes with ATS and complement. The ATS from the two rabbits showed almost the same antibody titer.

The cells of the thymus and bone marrow of five adult cattle were stained with ATS. Positive percentages were 99.5–100% (average: 99.8%) in thymocyte, while it was only 0–0.5% (average: 0.2%) in bone marrow cells. PBL were fractionated by E, EA and EAC rosette techniques or on nylon wool columns, and each cell fraction was stained by ATS and SIg (Table 1). By E rosetting, there was a slight increase in ATS positive cells, while it decreased in the non-rosetting cells. In EA and EAC rosetting cells, there was a marked increase in SIg positive cells, but it decreased in non-rosetting cells. By nylon wool columns, there were remarkable increases in the SIg positive cells in the adherent fraction and in the ATS potitive cell in the non-adherent fraction.

The PBL were treated with ATS and complement. The residual cells after this treatment were stained with SIg and

Table 1. Frequency of T and B lymphocyte in peripheral blood lymphocytes separated by rosette techniques ans nylon wool columns*

Treatment	E rosette**		EA rosette**		EAC rosette**		Nylon wool column	
	T(%)	B(%)	T(%)	B(%)	T(%)	B(%)	T(%)	B(%)
Unseparated cells	68.9***	27.1	71.0	23.9	62.0	26.2	67.1	24.6
Rosetting cells	79.3	19.2	1.5	96.0	22.4	69.4	—	—
Non-rosetting cells	46.3	49.5	80.8	9.5	73.1	18.3	—	—
Adherent cells	—	—	—	—	—	—	13.4	82.4
Non-adherent cells	—	—	—	—	—	—	80.4	9.6

* T lymphocytes were identified by indirect FA using rabbit anti-bovine thymocyte serum and FITC goat anti-rabbit IgG serum. B lymphocytes were identified by direct FA assay using FITC rabbit anti-bovine IgG serum.

** These lymphocytes were obtained from each rosette technique and nylon wool column.

*** Mean percent. One or two cattle were used for the rosette forming techniques and 4 cattle were used for the nylon wool columns.

Table 2. Treatment of lymphocyte with ATS and complement.

Animal No.	Treatment	% of cells positive		Stimulation index	
		T*	B*	PHA-P	Con-A
1	Non-treated	66.5	23.8	38.0	96.5
	ATS+complement	0	99.1	0.9	1.0
2	Non-treated	57.5	28.5	ND**	ND
	ATS+complement	0	99.5	ND	ND

* Identification of T and B lymphocytes were performed as shown in Table 1.

** Not done.

ATS and used in the stimulation test by mitogens (Table 2). The surviving cells after treatment with ATS and complement consisted of more than 99% SIg positive cells, while all of the ATS positive cells disappeared. In the simulation test by mitogens, the stimulation index was completely inhibited by the serviving cells. These results indicated that cells stained positively with ATS are those which possess a T lymphocyte antigen. Therefore, in the subsequent experiments, ATS positive cells were considered to be T lymphocytes and SIg positive cells B lymphocytes.

Identification of bovine lymphocyte populations by ATS and SIg:

The frequencies of T and B lymphocyte in the PBL, spleens and lymph nodes of 21 cattle were investigated (Table 3). As can be seen in the table, 89.8% of PBL, 92.1% of spleen cells and 87.6% of lymph node cells were identified as T or B lymphocytes. The remaining cells were other rypes of non-T or non-B cells.

Discussion

A method for the measurement of bovine T lymphocytes has been desired and the authors prepared ATS for this purpose. The specificity of the ATS was confirmed by the following observations. 1) Bone marrow cells did not react but 99.8% of the thymocytes reacted with ATS. 2) T-rich or B-rich fractions were obtained from PBL by E, EA and EAC rosette techniques and nylon wool col-

Table 3. Frequency of T and B Lymphocyte in Clinically Normal Cattle*

Cell source	T lymphocyte (%)		B lymphocyte (%)	
	Mean±SD	Range	Mean±SD	Range
PBL	64.4±6.4	54.0–77.5	25.4±3.9	20.9–31.0
Spleen	43.4±4.8	37.6–54.9	48.7±4.7	42.6–56.5
Lymph node	59.4±6.8	48.0–66.5	28.2±5.6	22.5–43.8

* Identification of T and B lymphocytes was performed as shown in Table 1. Healthy adult Japanese shorthorn, Japanese Black and Holstein Friesian cows (n=21), between 2 and 8 years old, were used.

umns. The fractionated cells were FA stained by ATS and SIg, and the results were satisfactory. 3) Treatment of PBL with ATS and complement resulted in the abrogation of blastogenic reactivity of lymphocytes to mitogen stimulation, and ATS-reactive cells were also eliminated by FA. The ATS was prepared as an antigen from the thymocytes of two JS cattle. This ATS showed good results not only when used in JS cattle but also in HF and JB cattle; there were no breed differences.

The percentages of the cells which did not react with SIg or ATS in the PBL, spleens and lymph nodes were 10.2, 7.9 and 12.4%, respectively. In future, the subpopulations of these non-T and non-B cells will have to be determined.

Recently, a similar study was reported [17]. In that report, the ATS was prepared by inoculating goats with calf thymocytes. Concerning ATS specificity, it was noted that ATS in non-reactive with bone marrow cells and reactive with thymocyte. The report also says that, there were 79.2±5.7% T lymphocytes and 26.9±4.4% B lymphocytes in the PBL. These values closely resemble to those obtained by the authors, although T cell percentage was slightly higher. Recently, a method to identify bovine T lymphocytes using heterologous T cell antiserum and peanut agglutinin has been reported [13]. According to this report, about 90% of PBL was identified as T or B cells. However, there has been no investigation of the spleen and lymph nodes.

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要　約

正常牛の末梢血，リンパ節，脾臓における T. B リンパ球の測定：中西　央・小山弘之・梶川　治・斉藤　博（北里大学獣医畜産学部獣医微生物学教室）——牛胸腺細胞を家兎に免疫して抗胸腺細胞血清（ATS）を作出し，これを牛の赤血球，肝臓粉末，骨髄細胞で順次吸収し間接蛍光抗体法によって牛 T リンパ球の同定を行った．21 頭の臨床的に正常な牛の末梢血（PBL），脾臓，リンパ節各々における T，B リンパ球の割合を ATS による間接蛍光抗体法と FITC 標識家兎抗牛血清による直接蛍光抗体法によって測定した．その結果，T，B リンパ球の割合は PBL で各々 66.4 ±6.4 %，25.4 ±3.9%，脾臓では 43.4 ±4.8%，48.7 ±4.7%，リンパ節では 59.4 ±6.8%，28.2 ±5.6% であった．