Flow Cytometric Analysis of Phenotypes of Canine Red Blood Cells with FITC-Labeled *Clerodendron Trichotomum* Lectin

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ABSTRACT. Canine red blood cells were divided into a positive type (C type) and a negative type (c type) by a agglutination test with *Clerodendron trichotomum* lectin (CTL). Blood cells changed from c to C type after suffering from mammary tumor were named c_m . The c type blood cells treated with neuraminidase were named c_n . These red blood cells were studied with flow cytometry using a directly fluoresceinated CTL. Positive percents of C type, c_m type, and c_n type were 49.3%, 43.8% and 81.0% respectively. While C showed one peak in histogram, c_m showed two peaks. The positive peak in the c_m blood cells suggested an appearance of a new blood cell population with a novel sugar chain structure after suffering from the tumor.—**KEY WORDS:** flow cytometry, lectin, tumor.

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Since 19th century, it has been known that certain plant extracts agglutinate red blood cells. It has been called a lectin, phytohemagglutinin or plant agglutinin [16]. Lectins are polypeptides that distinguish certain structure of sugar chains. Those have been used to detect various sugar chains on the cell surface membranes and to classify blood group antigens [9, 12]. Yoshida reported that *Clerodendron trichotomum* lectin (CTL) agglutinated canine red blood cells [4, 23]. We classified canine blood type into C type (agglutinated) and c type (non-agglutinated) by the agglutination test using CTL. And, the c type blood cells were agglutinated by CTL after treatment with neuraminidase and the treated cells were named c_n type [19].

Some kinds of blood group antigens are expressed as glycolipids or glycoproteins in various tissues and fluids. It has been reported that the synthetic pathways of carbohydrate chains of glycolipids and glycoproteins in carcinoma tissues are different from those in normal tissues. It has been shown that various alternation occurred on blood group antigens of erythrocytes and tissues in the process of oncogenesis [2]. We also reported that canine c type blood cells changed the character of agglutination with CTL, after the host suffered from mammary gland tumor. The blood type was named c_m type [20].

Because the agglutination test with CTL was quite qualitative but was not quantitative, we could not find any difference between C type and c_m type by the test. Then we planned to determine the quantitative differences among C type, c_m type and c_n type red blood cells by flow cytometry (FCM). A FCM analysis with FITC-conjugated CTL revealed the difference between C type and c_m type blood cells and suggested the alternation of the CTL epitope after suffering from a disease.

Cell preparation: Following dogs were prepared for this study. The C type dog was a Pomeranian, 2 years old, female. The c type dog was a mongrel, 2 years old, female. The c_m type dog was a Maltese, 4 years old, female. Each type of cells was collected by venipuncture and washed three times with physiological saline solution. The blood cells with c_n type were prepared by treatment with neuraminidase (Sigma, U.S.A.). The neuraminidase treatment was accomplished in the following manner: One ml of packed canine c type blood cells was incubated with

an equal volume of a neuraminidase solution (66 mU/ml of a 1/15 M phosphate buffer solution, pH 7.3, in 0.15 M NaCl) for 20 min at 37°C, and were washed five times with physiological saline solution.

Purification of CTL: The seeds of CT were collected in Nasu Country of Tochigi Prefecture, Japan. CT seeds (1 g) were dried well, and homogenized in 10 ml of physiological saline solution. The homogenate was kept overnight at 4°C, and then centrifuged at 1,000 g for 10 min. The supernatant was sterilized by filtration with Millex-GV (Millipore Corporation, Japan). The agglutination titer of the extract was 1:256 dilution to the C type blood cells. The crude CT extract was dialyzed for 20 hr against 20 mM Tris-HCl (pH 8.0) and was concentrated using the centrifugal concentrator cc-101 and the low temperature trap unit Tu-040 (Tomy, Japan). The extract was loaded on anion exchange high performance liquid chromatography using Model-342 gradient liquid chromatographic system (Becman, U.S.A.) with DEAE-5PW separation column (Toso, Japan), preequilibrated with 20 mM Tris-HCl buffer (pH 7.5). The CTL was eluted out with a linear gradient of NaCl from 0 to 0.5 M in 20 mM Tris-HCl (pH 7.5) at a flow rate of 0.5 ml/min. Each fraction was tested by a hemagglutination test with the C type blood cells. The active fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [7] with staining of Coomassie brilliant blue R-250. The hemagglutinative activity of the band was detected using red blood cell adherence assay by the methods of Towbin *et al.* [18] and Omi *et al.* [13].

FITC-labeling of CTL: CTL was labeled with fluorescein isothiocyanate (FITC; Sigma, U.S.A.) by the method proposed by Kitagawa [6]. The active fractions eluted from anion-exchange HPLC were condensed by Centrifo (Amicon, U.S.A.) to 1 ml and was dialyzed against 0.1 M NaHCO_3 (pH 9.6) for 20 hr. CTL was reacted at 4°C for 2 hr with 30 μl of FITC (1 mg/ml, in dimethyl sulfoxide). The reaction buffer was loaded on gel filtration using Sephadex G 25 column (Pharmacia, Sweden), which was equilibrated with 0.02 M PBS (pH 7.4, 0.15 M NaCl) containing 0.02% sodium azide. Absorbance of the gel filtrated fraction was determined at 280 nm and 495 nm.

FCM analysis: Flow cytometric analysis was performed

by the method of Hashimoto *et al* [3]. The C type, the c type, the c_n type and the c_m type blood cells were washed twice in a 0.06 M PBS (pH 7.3) and were resuspended in PBS as 0.3% cell suspension. The blood cells were incubated with the FITC-conjugated CTL at 37°C for 30 min. After washing twice in PBS, the cells were subjected to a FCM analyzer (Spectrum III; Ortho Diagnostics Systems Inc., NJ).

Purification of CTL: The crude CT extract was divided into 12 peaks by anion-exchange HPLC and the fractions at the eighth peak (Fr. 8) eluted in 51–53 min agglutinated the C type blood cells with a titer of 32 folds (Fig. 1). These active fractions were analyzed with SDS-PAGE. A single band with molecular weight 67 kd was detected on SDS-PAGE. Only the blotted band on polyvinylidene difluoride (PVDF) membrane attached the C and c_n type blood cells. It was indicated that the lectin of CT agglutinating C and c_n type cells is composed of a single molecule.

FITC-labeling of CTL: The purified CTL was subjected to FITC-labeling. Subsequently, the labeled CTL was separated from unreacted FITC by gel filtration. The absorbance peak of 280 nm and 495 nm were concomitantly filtrated at the 1st peak (Fig. 2). It was indicated that the lectin was sufficiently labeled with FITC and was separated from unreacted FITC. The agglutinative titer of the 1st peak was 16 folds.

FCM analysis: In histogram, the ordinate shows the number of analyzed cells, the abscissa log scale shows fluorescence intensity of each blood cell. Because there was no difference of histogram between FITC-conjugated CTL binding c type and FITC-unconjugated CTL binding c type blood cells, the c type blood cells were used as a negative control and set up a cut off point. The positive

Fig. 1. Purification of CTL by anion-exchange HPLC. The ordinate shows the time. The eighth fraction was used for FITC-labeling.

Fig. 2. FITC-labeling of CTL. The abscissa shows fraction number by filtration with Sephadex G-25. The ordinate shows absorbance at 280 nm (○) and 495 nm (●). The absorbance peak of 280 nm and 495 nm were concomitantly eluted out at the first peak.

percents of the C type, the c_m type and the c_n type cells were 49.3%, 43.8%, and 81.0%, respectively. Although there is no difference in positive cell percentage between C and c_m type blood cells, these cells showed an apparent difference in the positive and negative cell fluorescence intensity as shown in histogram. The C type had one peak and the c_m type had two peaks (Fig. 3).

FCM is useful to analyze antigenic determinants on the surface of red blood cells. We can explain the distinctions of blood cell surface sugar chains quantitatively and objectively by flow cytometric analysis.

In this experiment, positive percents of the C type, the c_m type and the c_n type were 49.3%, 43.8% and 81.0% respectively. There were significant differences in fluorescence intensity between C type and c_n type. The c_n type epitope was produced by releasing terminal sialic acids from carbohydrate chains of glycoconjugates on the c type erythrocyte membrane. The apparent differences of fluorescence intensity between C and c_n indicates a possibility of a carbohydrate epitope distinction between them. It was speculated that the c_n type epitope which had been masked by sialic acid on c type erythrocytes showed stronger affinity to CTL than C type epitope. The cell surface CTL-binding epitope has been thought to be restricted to the sugar chain terminal residue, beta-D-galactose [13]. A further explanation on biochemical character of CTL-binding epitope is desired.

On the other hand, positive percents of the C type and the c_m type were almost same, 49.3% and 43.8%, respectively. But the C type showed one peak, and the c_m type showed two peaks. The c_m type blood cells were thought to be consisted of two distinct groups. It was indicated that a novel blood cell group reacting with CTL was emerged after suffering mammary tumor. The positive peak in the c_m blood cells suggested the appearance of

Fig. 3. Flow cytometric analyses of the C antigen on erythrocytes using FITC-labeled CTL. The ordinate shows the number of analysed cells and the abscissa shows the fluorescence intensity of blood cell antigens. The c_m type was divided into two populations, reactive (+) and unreactive (-), by the fluorescence intensity.

a novel blood cell population with a novel sugar chain structure after the host suffered from the tumor. Two mechanisms were estimated for this phenomenon; the absorption of the novel carbohydrate epitope secreted from mammary tumor by erythrocyte membrane or an enzymatic modification of erythrocyte sugar chains by the tumor associated enzyme. Thomsen-Friedenreich (T) antigen on erythrocyte membrane which is normally occluded by sialic acid often appear in association with tumor [17]. The B5 antigen is a tumor-associated antigen on erythrocyte, which is thought to be absorbed from the serum [11].

Immunological studies of blood group antigens related with sugar chain antigens using lectin and monoclonal antibodies were performed in cases of colon carcinoma, pancreatic cancer, esophageal cancer and bladder cancer [1, 5, 8, 10, 14, 15, 21, 22]. In a veterinary oncological field, no immunological studies of blood group antigens have been carried out.

We could classify the C type and the c_m type by FCM using FITC-labeled CTL. This method will contribute to study novel antigens appeared in the oncogenesis.

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