**NOTE** *Immunology*

# Flow Cytometric Analysis of Canine Umbilical Cord Blood Lymphocytes

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ABSTRACT. Lymphocyte subsets in canine umbilical cord blood were flow cytometrically analyzed and compared with those of the dams' peripheral blood. The proportion of $CD3^+$ T lymphocytes, $CD21^+CD3^-$ B lymphocytes, and $CD3^-CD21^-$ non-T non-B lymphocytes in umbilical cord blood was 52.9%, 30.4%, and 16.7%, respectively. T lymphocyte/B lymphocyte ratio was significantly lower in the umbilical cord blood than in the dams' peripheral blood (2.1 ± 1.4 versus 11.0 ± 8.1, $P < 0.001$). In contrast, $CD4^+$ lymphocyte/$CD8^+$ lymphocyte ratio was significantly higher in the umbilical cord blood than in the dams' peripheral blood (7.6 ± 2.2 versus 1.8 ± 0.6, $P<0.001$). These findings clarified the phenotypic characters of canine umbilical cord blood lymphocytes.

KEY WORDS: canine, lymphocyte, umbilical cord.

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The composition of leukocytes in canine immune tissues has been investigated using a series of monoclonal antibodies that detect surface antigens on canine cells [15]. The similarity of the canine and human immune systems with regard to the characteristics of the lymphocyte subsets in peripheral blood has been elucidated [5, 9]. In contrast, some peculiar features of the canine immune system in comparison to those of the human immune system have also been reported [9, 10, 15]. In human peripheral blood, CD4 molecules are strongly expressed on helper T lymphocytes and weakly on monocytes ; however, they are strongly expressed on both T lymphocytes and neutrophils in canine peripheral blood [9, 15].

Human umbilical cord blood is widely used in transplantation for bone marrow reconstitution in human patients suffering from acute leukemia or non-regenerative anemia because it is abundant in hematopoietic stem cells [1]. In humans, the compositions and functions of the umbilical cord blood lymphocytes differ from those of the adult peripheral blood lymphocytes [2, 6, 8, 12–14, 17]. The proportion of $CD45RA^+CD45RO^-CD62L^+$ T lymphocytes, naïve T lymphocytes, in human umbilical cord blood is higher than that in human adult peripheral blood [13]. Berthou *et al.* revealed that the cytokilling activity exhibited by human umbilical cord blood lymphocytes was lower than that exhibited by human adult peripheral blood lymphocytes because the former lacked constitutive perforin expression [2].

The phenotypic characters of canine umbilical cord blood lymphocytes are entirely unclear. In the present study, we used flow cytometry to investigate the composition of canine umbilical cord blood lymphocytes and to compare it to that of the dams' peripheral blood lymphocytes.

We analyzed umbilical cord blood of 41 neonates, by one to three neonates per one parturition, which were born by cesarean section from 20 dams (6 beagles, 5 chihuahuas, 4 Scottish terriers, and 2 Yorkshire terriers, 1 bulldog, 1 miniature dachshund, and 1 Pekingnese). The dams' age at parturition was 1–8 years old. The cesarean sections were performed under anesthesia with intravenous injections of propofol followed by the inhalation of sevoflurane at 56–64 days after mating or artificial insemination. Umbilical cord blood was collected from the umbilical vein immediately after clamping the cord. The dams' blood was collected from the jugular vein just prior to the anesthesia. Sodium heparin was used as an anticoagulant.

In addition, there was no abnormal finding in physical examinations and data on complete blood count and/or urinalysis of all dams. All neonates using in this experiment were clinically healthy during the observation period, at least 3 hr after birth.

Fluorescent staining of the lymphocytes was performed as follows. Prior to staining the blood cells, the anti-canine CD45 mouse-IgG1 antibody (CA12.10C12; Serotec, Oxford, U.K.) was biotinylated using the Zenon Biotin-XX mouse IgG1 labeling kit (Invitrogen, Carlsbad, U.S.A.) according to the manufacturer's instructions. Subsequently, the prepared biotinylated anti-CD45 antibody was used with the following pairs of antibodies: anti-canine CD3 fluorescein isocyanate-conjugated (-FITC) antibody (CA17.2A12; Serotec) and anti-human CD21 phycoerythrin-conjugated (-PE) antibody (B-ly4; Becton Dickinson, Franklin Lakes, U.S.A.), and anti-canine CD4-FITC antibody (YKIX302.9; Serotec) and anti-canine CD8α-PE antibody (YCATE55.9; Serotec). The same procedure was used for negative stain-

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ing by fluorescent isotypic controls (Beckman Coulter, Fullerton, U.S.A.). The heparinized blood (50 $\mu l$) was reacted with each of the above-mentioned pairs of antibodies for 15 min at room temperature. It was mixed with 1.5 m*l* of an erythrocyte lysing solution (0.83% ammonium chloride, 0.1% potassium hydrocarbonate, and 0.004% ethylene diamine tetraacetic acid (EDTA) disodium salt in distilled water), and the sample was then left undisturbed for 15 min at 37°C as Kerttula *et al.* [7]. After centrifugation at 4°C, the supernatant was removed by aspiration. The pellet was washed twice with a wash buffer (phosphate buffered saline containing 1% bovine serum albumin, 2 mM EDTA disodium salt, and 0.03% sodium azide) at 4°C. Each sample was reacted on ice with R-Phycoerythrin-cyanine-5 conjugated streptavidin (Dako Japan, Kyoto, Japan) for 10 min. The samples were washed twice and then fixed in 1% paraformaldehyde solution for flow cytometric analysis.

The fluorescence of the stained cells was detected using a FACSCalibur flow cytometer (Becton Dickinson) and was analyzed using the commercially available CellQuest software (Becton Dickinson) as previously described [10].

We separated the lymphocyte-rich fraction from the leukocytes by gating on the forward and side scatter dot plot as shown in Fig. 1. The fraction was contaminated with considerable cellular debris, presumably produced by erythrocyte-lysis (data not shown). Therefore, we analyzed the $CD45^+$ cells in this fraction as lymphocytes.

All experimental values were expressed as mean and standard deviation. Mann-Whitney's *U*-test was used to determine statistical significance. $P<0.05$ was considered statistically significant.

The proportion of $CD3^+$ T lymphocytes, $CD21^+CD3^-$ B lymphocytes, and $CD3^-CD21^-$ non-T non-B lymphocytes in the umbilical cord blood was 52.9 ± 13.9%, 30.4 ± 9.8%, and 16.7 ± 8.6%, respectively, while in the dams' peripheral blood, it was 83.3 ± 8.0%, 12.1 ± 8.4%, and 4.6 ± 2.4%, respectively (Table 1). The ratio of $CD3^+$ lymphocyte/$CD21^+CD3^-$ lymphocyte, which represents the T lymphocyte/B lymphocyte ratio, in umbilical cord blood was significantly lower than that in dams' peripheral blood (2.1 ± 1.4 versus 11.0 ± 8.1, $P<0.001$) (Table 1).

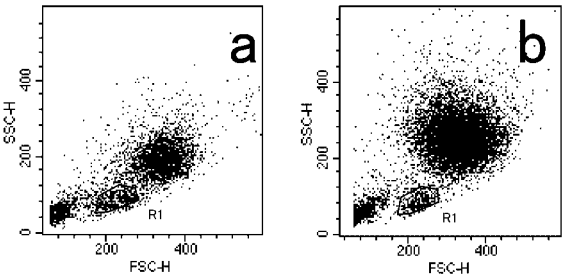


Fig. 1. Forward scatter (FSC) versus side scatter (SSC) dot plot of erythrocyte-lysed umbilical cord blood (a) and dam's peripheral blood (b). The cells gated by R1 in the FSC-SSC dot plot were analyzed as lymphocyte-rich fraction (a, b).

The proportion of $CD4^+(CD8^-)$, $CD8^+$ $(CD4^-)$, and $CD4^+CD8^+$ lymphocytes in the umbilical cord blood and the dams' peripheral blood are shown in Table 2. The ratio of $CD4^+$ $(CD8^-)$ lymphocyte/$CD8^+$ $(CD4^-)$ lymphocyte, which represents helper T lymphocyte/cytotoxic T lymphocyte ratio, in the umbilical cord blood was significantly higher than that in the dams' peripheral blood (7.6 ± 2.2 versus 1.8 ± 0.6, $P<0.001$)(Table 2).

T lymphocytes can be divided into 2 major subsets: $CD4^+$ $(CD8^-)$ helper T lymphocytes and $CD8^+$ $(CD4^-)$ cytotoxic T

Table 1. Percentages of $CD3^+$ T lymphocytes, $CD21^+(CD3^-)$ B lymphocytes, and $CD3^-CD21^-$ non-T non-B lymphocytes in the canine umbilical cord (n=41) and the dams' peripheral blood (n=20)

| Phenotype | Umbilical cord (%) | Dams' peripheral (%) | Mann-Whitney's *U*-Test |
|---|---|---|---|
| $CD3^+$ lymphocytes | 52.9 ± 13.9 | 83.3 ± 8.0 | P<0.001 |
| $CD21^+(CD3^-)$ lymphocytes | 30.4 ± 9.8 | 12.1 ± 8.4 | P<0.001 |
| $CD3^-CD21^-$ lymphocytes | 16.7 ± 8.6 | 4.6 ± 2.4 | P<0.001 |
| $CD3^+/CD21^+(CD3^-)$ ratio | 2.1 ± 1.4 | 11.0 ± 8.1 | P<0.001 |

All values are expressed as mean ± S.D.
The differences between the groups are evaluated by Mann-Whitney's *U*-Test.
NS: nonsignificant.

Table 2. Phenotypes on T lymphocytes in the canine umbilical cord blood (n=41) compared with the dam' peripheral blood (n=20)

| Phenotype | Umbilical cord (%) | Dams' peripheral (%) | Mann-Whitney's *U*-Test |
|---|---|---|---|
| $CD4^+(CD8^-)$ lymphocytes | 40.9 ± 11.0 | 45.7 ± 8.0 | NS |
| $CD8^+(CD4^-)$ lymphocytes | 5.7 ± 1.7 | 26.2 ± 6.4 | P<0.001 |
| $CD4^+CD8^+$ lymphocytes | 0.3 ± 0.2 | 1.5 ± 1.6 | P<0.001 |
| $CD4^+/CD8^+$ ratio | 7.6 ± 2.2 | 1.8 ± 0.6 | P<0.001 |

All values are expressed as mean ± S.D.
The differences between the groups are evaluated by Mann-Whitney's *U*-Test.
NS: nonsignificant.

lymphocytes. The proportion of these subsets in the peripheral blood of mature dogs was very similar to that in human blood [5, 9]. The results of this study revealed that the relative number of $CD8^+$ ($CD4^-$) lymphocytes was considerably lower in the umbilical cord blood than in the dams' peripheral blood, while there was no significant difference in the relative number of $CD4^+$($CD8^-$) lymphocytes (Table 2).

Harris *et al.* reported that the human CD4/CD8 ratio was slightly higher in umbilical cord blood than in adult peripheral blood (2.4 versus 1.7) [6]. This finding was supported by other similar studies on humans [14, 17]. The cytotoxic activity of human umbilical cord blood lymphocytes against alloantigenic cells was reported to be relatively low [1]. The reasons for this observation were considered to be decreased perforin expression in T lymphocytes [2], few cytotoxic effector T lymphocytes [12], and low interferon-gamma production, which is indispensable for the extensive proliferation of cytotoxic effector T lymphocytes [8].

The umbilical cord blood passed through the fetoplacental barrier facing the alloantigenic maternal cells [16]. Therefore, depression in the functional activity of fetal cytotoxic lymphocytes is considered to be beneficial for maintaining human pregnancy [16].

Dogs form an endotheliochorial type of placenta, which is a type of decidual placenta [3]. The placental, anatomical barrier between the fetal blood and maternal tissue in dogs is thin compared with that in species having non-decidual placentas [3].

The effect of sevoflurane on the proportion of the lymphocyte-subsets in canine umbilical cord blood needs further study, because some previous reports showed the immunomodulatory influences of general anesthesia [4, 11].

We speculated that the number of cytotoxic T lymphocytes in canine fetal blood was depressed for keeping immunological tolerance against maternal alloantigens. However, a more detailed cytological analysis of the umbilical cord blood lymphocytes is necessary for determining the precise reason for these results.

In conclusion, the present study clarified the composition of canine umbilical cord blood lymphocytes, which differed from those of the dams.

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