NOTE Internal Medicine

Relationship between Diarrhea and Peripheral Leukocyte Population in Neonatal Japanese Black Calves

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ABSTRACT. Neonatal Japanese Black (JB) calves show a high incidence of diarrhea. The objective of this study was to analyze the immune cell populations of neonatal JB calves in detail and examine its correlation with the incidence of diarrhea immediately after birth. Understanding the immune cell populations is helpful in clinics in order to determine the condition of the immune system for prevention of diseases. Blood samples were obtained from JB calves on the day of birth. The peripheral leukocyte populations were analyzed separately for calves that had diarrhea within 2 weeks after birth (diarrhea group; n=26) and for calves without diarrhea (control group; n=74). The numbers of the peripheral blood CD3+ CD4+ and CD8+ T cells were significantly lower in the diarrhea group compared with the control group. These findings suggest that the congenital lower peripheral γδ and CD8+ T cells results in a high risk of diarrhea in neonatal JB calves.

KEY WORDS: diarrhea, Japanese Black calf, leukocyte population.

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Neonatal calves are exposed to a large number of infectious microorganisms, including viruses, bacteria and parasites, immediately after birth. There is a high incidence of infectious diseases in calves, resulting in swelling of joints, diarrhea, pneumonia or meningitis in their early lives. Neonates showed substantially lower percentages of IFN-γ-producing CD4+ and CD8+ cells and a negative correlation between gestational age and IFN-γ-producing CD4+ cells. The Thymectomy does not cause immediate immunodeficiency until after birth [4, 5]. Neonatal immune responses have been extensively studied in murine models, and these studies have shown lower cell proliferation and cytokine responses to stimuli at birth than in adulthood [1, 15, 16]. There is a paucity of research that has evaluated immune status in fetal JB calves at birth. Therefore, in order to clarify whether a lower peripheral leukocyte population already existed before the onset of diarrhea in neonatal JB calves, peripheral leukocyte populations were analyzed on the day of birth.

One hundred JB calves housed on one farm in Aomori, Japan, were examined in this study. Artificial suckling was applied to all calves 3 to 5 days after birth. Twenty-six calves developed diarrhea within 2 weeks after birth (diarrhea group), and the other 74 calves did not show diarrhea during this period (control group). Ten calves in the diarrhea group and 5 calves in the control group were the 1st calves of their dams. Ten out of 26 calves in the diarrhea group and 41 out of 74 calves in the control group were male. Depression, dehydration and white watery feces were observed in the diarrhea group during the sick period within 2 weeks after birth, and these animals were treated with transfusion of Ringer and glucose solutions and injection of antibiotics. All diseased calves recovered within 3 to 5 days after treatment. We observed unexpected fever in some calves in this group, but watery diarrhea did not recur after treatment. The watery diarrhea was not observed in any calves after 2 weeks. There were no cases of dystocia, and...
the peripheral leukocyte populations of all calves were examined on the day of birth after consuming sufficient colostrum. Body weights of all calves were recorded on the day of birth.

Blood samples were collected into 1 tube with dipotassium-EDTA. The total number of the white blood cells (WBC) was determined using a blood cell counter (Celltac MEK-6358, Nihon Kohden, Tokyo, Japan).

Leukocytes surface receptors were determined using single or 2-color staining of peripheral blood mononuclear cells using the specific antibodies listed in Table 1. Leukocytes were isolated from 2 ml of blood after lysing red blood cells by adding 4 ml of 0.83% ammonium chloride solution. After hemolysis and washing with PBS (pH = 7.2), WBCs were resuspended in cold PBS. WBCs (1 × 10^6) were incubated with monoclonal antibodies at 4°C for 60 min. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgM and phycoerythrin-conjugated goat anti-mouse IgG (ICN Biomedicals, Costa Mesa, California, U.S.A.) at 4°C for 30 min. After washing the labeled WBC with PBS, the flow cytometric analysis was performed using a FACSscan flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, U.S.A.). Data from 10000 events per sample were analyzed using the Cell-Quest software (BD).

Statistical analysis was performed using the Mann-Whitney test and correlation among the data of each parameter in order to find the differences between the 2 groups using the Pearson’s test. The differences between groups were considered significant at P<0.05.

The body weights of male (30.7 ± 1.1 kg) and female calves (26.5 ± 0.7 kg) in the diarrhea group were significantly lower than those of male (33.5 ± 0.8 kg) and female calves (29.6 ± 0.7 kg) in the control group, respectively (P<0.05). The number of WBCs was lower in the diarrhea group (90.0 ± 6.9 × 10^9/μl) than in the control group (110.7 ± 4.4 × 10^9/μl). The calves in the diarrhea group had lower numbers of CD3+TcR1-N12+, CD8+ T cells and CD14+ cells (Table 2). There was no significant difference in CD4+ T cells. A significant positive correlation was observed between body weight and peripheral CD3+TcR1-N12+ T cell (R=0.39, P<0.05) and CD8+ T cell (R=0.36, P<0.05) numbers in this study. There were no correlations between the numbers of other leukocyte subsets and body weight.

We reported previously that the numbers of peripheral γδ and CD8+ T cells remained low in JB calves with WCS in the 1st month after birth [13], but it was not clear whether the numbers of these T cells were lower on the day of birth. In the present study, lower numbers of peripheral γδ and CD8+ T cells were observed at birth in the calves that developed diarrhea within 2 weeks after birth. A lower number of T cells seemed to be associated with the incidence of diarrhea in JB calves.

Tissue-specific accumulation of γδ T cells occurs in response to infection. Neonatal γδ T cells are functionally different from the majority of adult γδ T cells and display a distinct TCR repertoire and accessory molecule profiles. A previous study showed that γδ T cells obtained from cord blood had weak cytolytic activity in a tumor cell killing assay [11]. Cytotoxic CD8+ T cells are the main cells that kill virus infected cells, and a decrease in this subset of cells would diminish the ability to control viral infection, as reported in other animal models [17]. Infants with a severe clinical respiratory syncytial virus infection exhibited significantly lower CD8+ and CD8+/CD25+ (activated) T cell counts in the acute phase of illness [6], and the number of peripheral CD8+ T cells in infants with severe disease was significantly lower compared with those in infants with milder forms of illness [2]. γδ or CD8+ T cells represent important effector cells in the immune system, and lower numbers of these T cells in the diarrhea group might indicate that those neonates are more immune-suppressed.

The calves in the diarrhea group had significantly lighter body weights and a higher of primiparous dams. Neonatal calves largely depend on the dams for their physical development prior to calving. All muscle tissue, nerve fibers and energy reserves present in the calf are dependent on the

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**Table 1. Monoclonal antibodies against bovine leukocyte antigens used for flow cytometric analysis**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
<th>Isotype</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>MM1A</td>
<td>IgG1</td>
<td>Pan T cell</td>
<td>VMRD</td>
</tr>
<tr>
<td>CD4</td>
<td>CACT138A</td>
<td>IgG1</td>
<td>Helper/inducer</td>
<td>VMRD</td>
</tr>
<tr>
<td>CD8</td>
<td>BAT82A</td>
<td>IgG1</td>
<td>Cytotoxic</td>
<td>VMRD</td>
</tr>
<tr>
<td>TcR1-N12</td>
<td>CACT61A</td>
<td>IgM</td>
<td>γδ T cell</td>
<td>VMRD</td>
</tr>
<tr>
<td>MHC class II</td>
<td>CAT82A</td>
<td>IgG1</td>
<td>B cell / Monocyte</td>
<td>VMRD</td>
</tr>
<tr>
<td>CD14</td>
<td>MY4</td>
<td>IgG2b</td>
<td>Monocyte</td>
<td>Coulter(2)</td>
</tr>
</tbody>
</table>

a) VMRD=VMRD, Inc. (Pullman, WA, U.S.A.).

b) Coulter=Coulter Immunology (Hailed, FL, U.S.A.).

**Table 2. Peripheral leukocytes numbers in the two groups**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Diarrhea group</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+TcR1-N12- (cells/μl)</td>
<td>546.1 ± 52.5</td>
<td>664.0 ± 57.1</td>
<td>0.482</td>
</tr>
<tr>
<td>CD3+TcR1-N12+ (cells/μl)</td>
<td>425.3 ± 65.0</td>
<td>721.8 ± 62.5</td>
<td>0.011</td>
</tr>
<tr>
<td>CD8+ (cells/μl)</td>
<td>125.6 ± 24.8</td>
<td>131.0 ± 10.4</td>
<td>0.832</td>
</tr>
<tr>
<td>CD8+ (cells/μl)</td>
<td>122.0 ± 19.9</td>
<td>199.9 ± 6.0</td>
<td>0.001</td>
</tr>
<tr>
<td>CD8+/CD8+ rate</td>
<td>0.96 ± 0.16</td>
<td>0.64 ± 0.05</td>
<td>0.292</td>
</tr>
<tr>
<td>MHC class-II/CD14+ (cells/μl)</td>
<td>119.1 ± 23.9</td>
<td>144.3 ± 11.5</td>
<td>0.545</td>
</tr>
<tr>
<td>CD14+ (cells/μl)</td>
<td>340.7 ± 55.2</td>
<td>531.8 ± 52.1</td>
<td>0.088</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SE.
nutritional status of the dam, and thus, it is highly likely that weak calves were born to dams in an undernourished condition [9]. In humans, T cell responses are immature at birth compared with adults [15], and premature neonates demonstrated a significantly lower number of T cells [7, 14]. In addition, development of peripheral T or B cells after birth was suppressed in JB calves that were born from dams with insufficient nutrition during the last period of gestation and calves born from primiparous dams [19]. Thymic hypoplasia associated with decreased immune function was reported in calves with stillbirth/perinatal WCS, and it was suggested that intrauterine growth retardation was caused by lack of growth factors during the fetal period [18]. Taken together, the results of the present study suggest a disturbance of T cell development during the last period of gestation, since this period can influence a peripheral leukocyte population in newborn JB calves.

The principal observation in the present study was lower numbers of peripheral γδ and CD8+ T cells in JB calves with diarrhea. This reduced immune condition could aggravate infectious diseases and may explain some of the immunologic abnormalities accompanied by diarrhea. The reason for no difference in CD4+ T cells between the 2 groups in this study is not clear. Children born very preterm had significantly lower CD4+ T cell percentages compared with healthy controls [20]. Potent immune activation in early life in JB calves might contribute to protection from bacterial infection. The ideal immune conditions for keeping JB calves healthy are still unclear. Further studies are needed in this field.

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REFERENCES