Age-Related Alterations in Peripheral Leukocyte Population of Healthy Holstein Dairy Cows during the Pre-Calving Period

Hiromichi OHTSUKA1)*, Masami UEMATSU2), Yumi SARUYAMA1), Maiko ONO1), Masayuki KOHIRUIMAKI3), Takaaki ANDO4) and Seiichi KAWAMURA1)

1) School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628, 2) Department of Yamagata Agricultural Mutual Relief Association, Yamagata 990–2171, 3) Kohiruimaki Animal Medical Service, Tohoku, Aomori 039–2683 and 4) School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Bunkyodai 069–8501, Japan.

(Received 16 December 2008/Accepted 8 April 2009)

ABSTRACT. Older cows show a high incidence of infectious diseases during the periparturient period. The periparturient infectious diseases are closely associated with the immune function of dairy cows during the pre-calving period. In order to evaluate the relationship between the immune cell population and age in the cows during the pre-calving period, we obtained blood samples from 170 dairy cows during the pre-calving period. We chose only healthy cows, which did not develop clinical diseases within 2 weeks after the calving in this study. The animals were divided into 4 groups based on their parity: in their 1st pregnancy (Group 1), in their 2nd pregnancy (Group 2), in their 3rd calving (Group 3) and in more than 3rd pregnancy (Group 4). The numbers of the peripheral blood CD3+TcR1-N12+ and MHC class-II+CD14– lymphocytes were significantly higher in Group 1 compared to Group 4. This result indicated that the lower γδT cells and B cells in older cows compared with heifer during pre-calving period.

KEY WORDS: dairy cow, leukocyte population, pre-calving period.

NOTE


There are high incidences of infectious diseases in the dairy cows during the periparturient period. Appearance of infectious disease after the calving is recognized to relate closely to natural impaired immune conditions from the pre-calving period. Because it is well known that lymphocyte or neutrophil numbers and their functions decrease markedly a few weeks before the calving [8, 17]. In addition, lower peripheral T cell numbers were observed previously in cows with inflammatory diseases by the day of calving, before the onset of disease [12]. Therefore we suspected that impaired immune condition in periparturient dairy cows with infectious diseases might be reflected in unstable changes in peripheral leukocyte populations during pre-calving period.

The incidence of infectious diseases such as mastitis or metritis is relatively higher in older cows [6, 13]. The macrophages or lymphocytes functions are known to decline along with ageing in animals [1, 4, 16]. Sakata-Kaneko et al. [18] analyzed the effect of ageing on the responsiveness of human CD4+ T cells. They reported a decrease in IFN-γ production by lymphocytes obtained from old subjects compared to young controls [3, 7]. The reasons for the higher incidence of mastitis in the older cows may be due to a lower immune cell function by ageing especially during the pre-calving period. No reliable evidence has been reported for the association of age with the immune function in the healthy dairy cows before calving. In order to determine the relationship between the age and the number of leukocyte populations, we analyzed the populations and the proliferative ability of peripheral blood leukocytes in the healthy cows during the pre-calving period.

One hundred seventy clinically healthy Holstein dairy cows that did not develop clinical diseases within 2 weeks after calving were examined in this study. All cows were housed in tie-stall barns of 14 dairy farms in Aomori and Yamagata Prefecture, Japan. Cows were divided into four groups; in their first calving (Group 1, N=32, age; 1.99 ± 0.04 yr), in their second calving (Group2, N=57, age; 3.04 ± 0.03 yr), in their third calving (Group 3, N=33, age; 4.18 ± 0.06 yr) and in more than third calving (Group4, N=48, age; 6.22 ± 0.18 yr). All cows were used to analyze for leukocyte population during the pre-calving period only once, and samples were divided into three periods according to the time of bleeding: the Period I (60 to 41 d pre-calving), the Period II (40 to 21 d pre-calving) and the Period III (20 to 0 d pre-calving) (Table 1).

Blood samples were collected into two tubes, one with heparin and the other with dipotassium-EDTA (EDTA-2K). The total number of the white blood cells (WBC) was determined using EDTA-2K containing blood samples by a blood cell counter (PC607, ERMA, Germany). Two mL of EDTA-2K containing blood samples were mixed with 4 mL of 0.83% ammonium chloride solution to lyse the RBC. After the hemolysis and washing with PBS (pH 7.2), WBC were resuspended in cold PBS. Two-color flow cytometry was performed using these isolated WBC. WBC (1 × 10⁶) were incubated with monoclonal antibodies prepared in PBS at 4°C for 60 min. The primary antibodies and the description of the working solutions are presented in Table 2. After washing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC) labeled anti-mouse IgM and phycoerythrin (PE) labeled anti-mouse IgG1 (ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A.) at 4°C for 30 min. After washing the WBC with PBS, the flow cytometric
Stimulated with 10 μL of medium with 10% fetal bovine serum. Each sample was heparinized samples and seeded into 96-well microplates at blood mononuclear cells (PBMC) were separated from the using Cell Quest software (BD). The differences between groups were considered significant during the same time period using Tukey-Kramer’s test. The stimulated index rate of PBMC was calculated by the following formula as; Stimulation Index (SI)=(O.D. of stimulated sample)/(O.D. of control)

Animals were divided into three periods according to the date of sampling as well as the number of pregnancy: Period I: 60 to 41 d before calving, period II: 40 to 21 d before calving and the period III: 20 to 0 d before calving. Group 1 (first pregnancy), Group 2 (second pregnancy), Group 3 (third pregnancy) and Group 4 (more than third pregnancy). Data are expressed as mean ± S.E.

### Table 1. The number of animals, parity, and the date of sampling before calving

<table>
<thead>
<tr>
<th>Period</th>
<th>Animal number</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Date of sampling</td>
<td>12</td>
<td>17</td>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Animal number</td>
<td>10.6 ± 1.4</td>
<td>10.8 ± 1.2</td>
<td>10.2 ± 2.0</td>
<td>10.9 ± 1.2</td>
</tr>
<tr>
<td>II</td>
<td>Date of sampling</td>
<td>10</td>
<td>18</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Animal number</td>
<td>31.7 ± 1.5</td>
<td>29.9 ± 1.3</td>
<td>28.4 ± 1.2</td>
<td>29.9 ± 1.7</td>
</tr>
<tr>
<td>III</td>
<td>Date of sampling</td>
<td>22</td>
<td>13</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Animal number</td>
<td>50.4 ± 1.4</td>
<td>52.3 ± 1.0</td>
<td>50.5 ± 2.1</td>
<td>51.0 ± 1.2</td>
</tr>
</tbody>
</table>

The original concentration of the MAb solution was 1 μg/mL.

### Table 2. Antibodies used in the immunostaining of peripheral blood mononuclear leukocytes

<table>
<thead>
<tr>
<th>Antigen</th>
<th>MAb 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>CACT80C</td>
<td>IgG1</td>
<td>Cytotoxic</td>
<td>VMRD</td>
</tr>
<tr>
<td>CD14</td>
<td>MY4</td>
<td>IgG2b</td>
<td>Monocyte</td>
<td>Coulter</td>
</tr>
<tr>
<td>TcR1-N12</td>
<td>CACT61A</td>
<td>IgM</td>
<td>γδT-cell receptor</td>
<td>VMRD</td>
</tr>
<tr>
<td>MHC Class II</td>
<td>CAT82A</td>
<td>IgG1</td>
<td>Class II major histocompatibility complex</td>
<td>VMRD</td>
</tr>
</tbody>
</table>


Peripheral blood lymphocytes in cows decrease gradually toward calving before calving [8, 11]. Therefore most cows had decreased peripheral lymphocyte numbers during the pre-calving period. The leukocyte population in Group 4 was similar to Group 3 during all experimental periods, and this population was different from Group 1. The most noticeable finding in the current study was distinct age-associated differences in γδT cell and B cell.

Wilson et al. [20] described that the percentage of γδT cell was lower in older dairy cattle than in the young cattle. The percentage of γδT cell was the highest at birth and gradually decreased to the adult level by 150 days of age. γδT cells stimulated with non-peptidic phospho-antigens produce high levels of cytokines, mainly interferon (IFN)-γ and tumor necrosis factor (TNF)-α [14, 15]. Moreover, γδT cells are involved in coordinating the interplay between innate immunity and acquired immunity, and contribute to the differentiation of γδT cell responses toward either T helper cell type 1 or type 2 [5, 9]. The alteration of γδT cells could contribute to the age-related decrease in T cell-mediated immune responses.
4 compared to the Group 1 seemed to a peculiarity of leuko-
cytes and it may represent an immuno-senescence. 

CD3+ (× 10^7/µL) 
I 21.29 ± 2.32ab 22.68 ± 2.47ab 15.37 ± 2.30ab 15.53 ± 0.75ab 
II 19.78 ± 4.80 19.36 ± 2.80 16.93 ± 1.45 13.64 ± 2.10 
III 17.00 ± 2.02 18.96 ± 1.88 10.66 ± 1.39 13.78 ± 2.61 

CD3’TeR N12’ (× 10^7/µL) 
I 10.64 ± 0.46 14.43 ± 1.19 8.59 ± 0.78 10.21 ± 0.42 
II 9.37 ± 1.69 12.44 ± 1.66 11.08 ± 2.89 9.04 ± 0.74 
III 9.61 ± 1.12 11.91 ± 1.62 10.21 ± 2.15 11.62 ± 1.72 

CD3’TeR N12’ (× 10^7/µL) 
I 5.99 ± 0.61a 3.19 ± 0.16b 1.70 ± 0.17b 2.39 ± 0.20b 
II 4.87 ± 1.33a 2.91 ± 0.47b 1.81 ± 0.60b 1.83 ± 0.43b 
III 3.93 ± 0.64 2.69 ± 0.29 1.65 ± 0.42 1.78 ± 0.26 

CD4+ (× 10^7/µL) 
I 6.31 ± 0.35 8.17 ± 0.43 5.30 ± 0.55 6.25 ± 0.26 
II 6.11 ± 1.22 6.67 ± 0.86 6.07 ± 0.95 6.02 ± 0.72 
III 5.65 ± 0.52 6.64 ± 0.60 3.79 ± 0.41 6.66 ± 0.65 

CD8+ (× 10^7/µL) 
I 3.62 ± 0.12 5.59 ± 0.27 3.25 ± 0.37 3.40 ± 0.13 
II 4.33 ± 0.60 4.01 ± 0.55 4.52 ± 0.67 3.72 ± 0.66 
III 2.97 ± 0.35 3.71 ± 0.38 3.13 ± 0.28 3.65 ± 0.50 

CD14’ (× 10^7/µL) 
I 5.30 ± 0.84 7.73 ± 2.10 7.54 ± 1.09 4.12 ± 0.70 
II 7.29 ± 1.58 4.98 ± 0.69 6.74 ± 1.46 6.27 ± 1.01 
III 8.96 ± 1.31 6.57 ± 0.71 5.97 ± 1.27 6.15 ± 0.67 

MHC class-II’CD14’ (× 10^7/µL) 
I 13.23 ± 0.42ab 12.32 ± 0.54ab 9.18 ± 0.77ab 9.97 ± 0.29ab 
II 14.70 ± 1.96 13.56 ± 1.55 13.33 ± 1.79 9.20 ± 1.02 
III 16.31 ± 3.20 16.52 ± 1.84 9.57 ± 2.35 10.44 ± 1.00 

Proliferation by PHA (SI) 
I 2.84 ± 0.49 1.99 ± 0.31 1.85 ± 0.35 2.16 ± 0.31 
II 3.72 ± 0.69 2.09 ± 0.34 2.12 ± 0.51 1.76 ± 0.37 
III 2.74 ± 0.51 2.53 ± 0.31 2.36 ± 0.46 1.91 ± 0.33 

Proliferation by PWM (SI) 
I 2.19 ± 0.34 1.74 ± 0.24 1.81 ± 0.39 1.94 ± 0.24 
II 3.19 ± 0.53 1.93 ± 0.24 1.86 ± 0.38 1.63 ± 0.32 
III 2.49 ± 0.43 2.22 ± 0.27 1.93 ± 0.33 1.53 ± 0.19 

Data are expressed as the mean ± S.E. 
*Different letters indicate significant difference between groups within the same period (P<0.05). 
Period I (60 to 41 d pre-calving), the period II (40 to 21 d pre-calving) and the period III (20 to 0 d pre-calving).
III in this study, which might represent a lower function of T cells in older cows.

In the current study, we observed the decrease in γδT cell and B cells with the increase in parity of cows during the pre-calving period. These might be associated with the increased susceptibility of the older cows to infections compared with heifers. Special care of older cows is needed to maintain their immune function during the pre-calving period by providing a good environment.

ACKNOWLEDGMENTS. The authors are grateful for Prof. Oikawa and Dr. Takagishi in Kitasato University for his supporting this study. Additional appreciation also goes to all farm owners and farm personnel in the enrolled farms for their cooperation and interest in our research.

REFERENCES


