Effect of administering activated lymphocytes originated from the dam on the immune cell reaction in Holstein calves

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Abstract

Injection of lymphokine activated killer (LAK) cells is known as useful for activation of cellular immune system. Although the effect of LAK cells has been clarified in human or mice, this effect on function of immune cells has not been examined in calves. Healthy ten Holstein calves were injected with the LAK cells 2 days after birth (LAK Group), and another eight calves were observed as controls (Control Group). All calves received the colostrum formulation on the day of birth, and then, were inoculated with a live attenuated vaccine of bovine herpesvirus (BHV)-1 at 2 (the first vaccination) and 6 (the second vaccination) weeks after birth. Peripheral blood of their dam obtained 3 weeks before calving was used for preparation of LAK cells. Blood samples were taken prior to vaccine inoculation and 3 days after the first inoculation, as well as 3 and 6 days after the second vaccination from all calves. Numbers of CD8⁺ and CD21⁺ cells increased significantly after the second vaccination in the LAK Group compared with Control Group. The present study suggested the improved effect of injecting LAK cells originated from dams on immune cells function of young calves after BHV-1 live vaccine.

Key words: bovine herpesvirus, calves, dam, immune cells, lymphokine activated killer

Introduction

Ingestion of colostrum is important to ensure adequate passive immunity of the neonatal calves. Previous studies indicated that colostral immune cells from dam pass through the intestine into neonatal circulation (Volatilelli et al. 1990, Liebler-Tenorio et al. 2002). Passively transferred colostral cells may have antigen-specific memory characteristics, aiding in immediate pathogen clearance in a calf (Donovan et al. 2007). In addition, Langel et al. (2015) revealed a greater response to vaccines up to 6 to 10 months post-whole colostrum feeding when compared with cell-free colostrum. Maternal colostral leukocytes are suggested to have a positive influence on the development of immune system in the newborn calves (Reber et al. 2008).

In adoptive immunotherapy of humans, effector T
cells are used frequently for activation of cellular immune function. Lymphokine-activated killer (LAK) cells are known as useful for an adoptive immunotherapy which involves isolation of lymphocytes from blood of a patient and proliferating them in the presence of the interleukin-2 (IL-2) (Valteau-Couanet et al. 2002). Cytotoxic T cells scan the intracellular environment in order to target and destroy virus infected cells (Linn and Hui 2003). Therefore, LAK cells injection can be expected to induce activation of the adoptive cellular immune function in animals. Although colostral immune cells traffic into neonatal calves’ circulation (Liebler-Tenorio et al. 2002, Williams 1993), it is not clear whether maternal LAK cells injection might affect the activation of immune cells in young calves.

Bovine herpesvirus types 1 (BHV-1) is one of the causative agent of respiratory infection in calves, and this virus affects calves’ growth from their birth to approximately 18 months of age and can reach 100% of mortality in young cattle (Schudel et al. 1986). Campos et al. (1991) demonstrated that IL-2-activated peripheral blood mononuclear cell (PBMC) preferentially lyse BHV-1-infected cells, and these IL-2-activated effector cells were capable of significantly reducing virus propagation. Therefore, the present study was undertaken to evaluate the acquired immune function of the neonatal calves after injection of the LAK cells originated from PBMC of the dams by activation with IL-2 and anti-CD3 antibody. To evaluate changes in acquired immune responses of the neonatal calves with dam’s LAK cells injection, we observed the leukocyte population and cytokine RNA expression levels in PBMC following inoculation of available BHV-1 vaccine.

### Materials and Methods

Eighteen healthy Holstein calves kept in one herd were used in this study. All calves were given 2 x 225g of a commercially available dried colostrum formulation (Head Start; Bayer Ltd., Leverkusen, Germany) on the day of birth, and then all calves were given milk replacer. The calves were divided into two groups: ten calves were intravenously injected the LAK cells in 50ml of PBS(-) fluid 2 days after their birth (LAK Group), and the other eight calves, which received only 50ml PBS(-) at 2 days of age, were used as controls (Control Group). Blood samples were collected from the jugular vein on the day of birth and 3, 7, 14, 17, 42, 45 and 48 days after birth. The samples were collected in tubes containing dipotassium-EDTA for analysis of leukocyte population and in tubes containing heparin for analysis of cytokine production. Serum samples were taken for measurement of BHV-1 antibody titer. White blood cell (WBC) counts were determined with a blood cell counter (Celltac MEK-6358, NIHON KOHDEN, Tokyo, Japan). The procedures used in the present study were in accordance with the principles and guideline for animal use set by the Guidelines for Institutional Laboratory Animal Care and Use of the School of Veterinary Medicine at Kitasato University, Japan. All calves were kept in a pen with a space of more than 1.5 m² per animal and with straw bedding into a better-ventilated area, and fed two times milk drink in a day during the experimental period.

Twenty ml of peripheral blood were collected from dams 3 weeks before calving for preparation of the LAK cells, and separated PBMC were suspended in recombinant human (rh) IL-2-containing culture medium (LAM-1, Canine-Lab, Tokyo, Japan) with 2.5% fetal bovine serum. The bottom of a 250 ml flasks were used for cell suspension culture (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) in the plates coated with 5 μg/ml anti-bovine CD3 antibody solution diluted with Dulbecco’s PBS (D-PBS; Nissui Co., Tokyo, Japan). The cell suspension was cultured in the flask coated with ant-CD3 antibody at 37°C under 5% CO₂. On days 3 and 6, 20 ml of culture medium containing rhIL-2 (LAM-2, Canine-Lab, Tokyo, Japan) was added. On day 7, the cell suspension was transferred to a culture bag with culture medium containing rhIL-2 (LAM-3, Canine-Lab, Tokyo, Japan) from the flask. On day 14, the cultured cells were harvested from the bag and washed twice with sterile physiological saline. Finally, the cells were suspended in 50 ml of sterile physiological saline and filtrated through a sterile 100 μm nylon

### Table 1. Lymphocyte population of LAK cells on Pre and Post culture (%).

<table>
<thead>
<tr>
<th>CD antigen</th>
<th>Pre culture</th>
<th>Post culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺</td>
<td>49.12± 5.12</td>
<td>93.21± 0.80</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>20.28± 4.39</td>
<td>18.88± 5.3S</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>17.40± 3.04</td>
<td>66.52± 5.50</td>
</tr>
<tr>
<td>CD21⁺</td>
<td>16.49± 2.90</td>
<td>1.37± 0.51</td>
</tr>
<tr>
<td>WC1-N12⁺</td>
<td>5.60± 1.40</td>
<td>8.69± 4.41</td>
</tr>
</tbody>
</table>

Means ± SE of the lymphocyte population.
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Table 2. Primary monoclonal antibodies used for flow cytometric analysis.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Ab Clone</th>
<th>Iso type</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>CACTI 38 A</td>
<td>IgG1</td>
<td>Helper/inducer</td>
<td>VMRD&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD8</td>
<td>CACT80C</td>
<td>IgG1</td>
<td>Cytotoxic</td>
<td>VMRD&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD14</td>
<td>MY4</td>
<td>IgGb2</td>
<td>Monocyte</td>
<td>BC&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD21</td>
<td>BAQ15A</td>
<td>IgM</td>
<td>B cell</td>
<td>VMRD&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD335</td>
<td>MCA2365EL</td>
<td>IgG1</td>
<td>γδ T cell</td>
<td>VMRD&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>WC1-N12</td>
<td>IL-A29</td>
<td>IgG1</td>
<td>B cell Monocyte</td>
<td>VMRD&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>MHC classIl</td>
<td>CAT82A</td>
<td>IgG1</td>
<td>B cell</td>
<td>VMRD&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> VMRD=VMRD, Inc. (Pullman, WA, USA)  
<sup>2</sup> – BC=Beckman Coulter (Miami, FL, USA)  
<sup>3</sup> – Serotec=AbD Serotec, Int., Ltd (Oxford, UK)

Stained cells were determined using Flow Jo software (Tree Star, Inc. Oregon, USA). The number of each population was calculated by multiplication of the percentage with the WBC counts.

To analyze the cytokines mRNA expression of lymphocytes, PBMC were collected and seeded into 24 well microplates at 5×10<sup>6</sup> cells/well at a final volume of 1 ml/well in RPMI 1640 medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 2-mercaptoethanol (2-ME) and 10% fetal calf serum. Each sample was cultured with 10 μg/ml of phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO, USA). After incubation for 12 hr at 37°C, the supernatants were removed and cells were resuspended using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Analysis of cytokine mRNA levels was performed by a real-time PCR as described previously (Maeda et al. 2013). The target DNA sequences were specifically amplified using the primers as previously designed from bovine β-actin (forward 5’ CTTTTACAACGAAGCTCGTG 3’; reverse 5’ CACGTTCGTTAGGATCCTTC 3’ Acc. No.; K00622), bovine IL-2 (forward 5’ TTGATCTCTCTGGGGTTCAG 3’ Acc. No.; M12791), bovine IL-4 (forward 5’ TGCCCCAAAGAACACAACTG 3’; reverse 5’ TTAGCCTTCAAAGAGTC 3’ Acc. No.; M29867). Results are presented as Ct values, where Ct is the difference in threshold cycles for a target using β-actin as an internal control. Ct values define the threshold cycle of PCR, at which amplified products were detected. The mRNA expression levels of cytokines were calculated by the following formula: cytokine target mRNA = 2^(-(Ct value – β-actin Ct value))

Statistical analysis of each parameter was conduct-
ed using Mann-Whitney test comparing the two groups, and values of p<0.05 were regarded as significant. The mean values and standard errors of the clinical and laboratory data were calculated.

Results

Figure 1 shows changes in the peripheral T cell subsets of the two groups. The average numbers of CD4+ and CD8+ T cells per microliter of blood increased gradually in both groups, but significant differences in the number of these cells between the groups were not found after injection of LAK cells up to the first inoculation of BHV-1 vaccine. After the second vaccine inoculation, the average number of CD4+ and CD8+ T cells increased at day 3 in the LAK Group, and CD8+ T cell number was significantly higher compared to that in the Control Group (p<0.05). There was no significant difference in the number of WC1-N12+ T cells between the two groups, and there were no changes in this cells population in the two groups after vaccine inoculation.

In both groups, CD14+ cell number increased between days 0 and 3, and this cell population remained stable after LAK cell injection. The time-course changes in the peripheral CD335+ cells showed an increasing trend with regard to their numbers in the two groups. The number of CD335+ increased after the first and the second vaccine inoculations in the LAK Group, but a significant difference was not found. The average number of CD21+ and MHC class-II+CD14+ cells increased gradually in both groups after birth, but no significant difference was found between the two groups until the second vaccine inoculation. In the LAK Group, num-
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of IFN-γ, IL-2 and IL-4 reached the peak after the second vaccination in the LAK Group, and IFN-γ expression in the LAK Group was higher than that in the Control Group at 45 days of age (p = 0.06) (Fig. 3).

The antibody titer against BHV-1 was the highest at day 0, and the titer decreased markedly after the first vaccine inoculation in both groups (Fig. 4). Significant difference was not found between the two groups.

### Discussion

To our knowledge, this study is the first report describing the improvement of booster reaction of lymphocytes such as enhancement of lymphocyte numbers and cytokines levels with vaccine inoculation of attenuated BHV-1 live vaccine in the calves injected with LAK cells during their neonatal period. The effect of LAK is suggested to be depending on the adhesion molecules or signals in the cells, and avidity of cytotoxic CD8⁺ T lymphocyte (CTL) during activation (Grimm et al. 1982). The promotion of the proliferation of lymphocytes after the second vaccine inoculation might be affected by the of LAK cells injection in the young calves.

The increase in immune cell numbers with IL-2 stimulation, specifically T cells and natural killer cells, will enhance an attack against any virus infected cells. In this study, peripheral CD8⁺ T cell number increased after the second inoculation of vaccine in the LAK Group. CD8⁺ T cells are fundamental for immune-mediated clearance of viral infections, and this type of T cells play a dominant role in the recovery of calves from virus infection (Taylor et al. 1995). Upon activa-
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...ion, effector memory T cells enter the circulation and peripheral tissues (Masopust et al. 2001, Jung et al. 2010). The degree of CD8$^+$ T cells reaction after the secondary infection depends on the quality and the quantity of memory CD8$^+$ T cells (Badovinac et al. 2002, Kaech et al. 2002). LAK injection might promote the expansion of the primary memory CD8$^+$ T cells, leading to increased numbers and sufficient representation of memory CD8$^+$ T cells at a single booster vaccination. Mouse and human LAK/NK cells are known to activate dendritic cells in vitro (Valteau-Couanet et al. 2002, Capobianco et al. 2006). Therefore, the maintenance of high avidity CTL in vivo has been a challenge when designing effective vaccines for viral infection.

In this study, CD4$^+$ cells number increased after the second inoculation of vaccine following increased CD8$^+$ cells in the LAK Group. CD4$^+$ T helper cells are well known for their role in providing critical signals during priming of CTL cells responses in vivo (Manzke et al. 2013). A simple activation of naive CD8$^+$ T cells requires the interaction with professional antigen-presenting cells, mainly matured dendritic cells. The dendritic cells have to interact with both CD4$^+$ and CD8$^+$ T cells to generate long lasting memory T cells and to allow repetitive stimulation of cytotoxic T cells (Hoyer et al. 2014). During this process, the CD4$^+$ helper T cells provide a potent activating signal to the naive CD8$^+$ T cells.

We observed an increased number of peripheral B cells, including CD21$^+$ cells and MHC class-II$^+$CD14$^+$ cells after intervention with a single booster vaccination in the LAK Group. In response to antigenic stimulation, some B cells differentiate into plasma cells that secrete antibodies, while the others become memory B cells (Rajewsky 1996). While the persistence of memory B cells is well documented, there is much less information as to the homing as well as short and medium term dynamics of memory B cells following infection or vaccination (Hendrikx et al. 2011). The rapid appearance of peripheral memory B cells was detected with re-stimulation of antigen in humans (Blanchard-Rohner et al. 2009). The increased number of peripheral B cells in the LAK Group in the current study resembles that reported previously, indicating distinct requirements for activation of memory B cells. LAK cells injection to neonatal calves may be effective for activation of memory response of B cells. On the other hand, increased antibody titers against BHV-1 was not found in the LAK Group. It has been described that the presence of passively acquired anti-BHV-1 antibodies from colostrum may inhibit the development of an active antibody response to BHV-1 infection (Lemaire et al. 1995). Previous investigation reported that increased BHV-1 antibody titer was not found after two times inoculation of BHV-1 live vaccine in young calves (Otomaru et al. 2013), which is similar to the results of obtained in the current study. Weak plasma cell and germinal centre B cell responses is one of the characteristic of innate and adaptive neonatal immune system in early life (Mohr and Siegrist 2016), productive function of vaccine-specific IgG antibody in neonates is lower than in older children (Michael and Pichichero 2014). Siegrist (Siegrist 2001) reported that the apparent impairment of CD4 and CD8 T cell function in early life is due to lower suboptimal antigen-presenting cells to T cells interactions. Therefore, nonreactive antibody production after inoculation in the neonatal animals seem to be due to weak and immature humoral immune cell function. However, the persistence of maternal antibodies may limit infant antibody responses, but induction of T cells responses is largely unaffected by these passively transferred antibodies (Siegrist 2001). Therefore, it was suggested even if the neonatal calves with maternal antibodies were injected with LAK cells, the promoting effect on lymphocyte proliferation was obtained. The injection of LAK cells might have not promoted the production of antibodies after inoculation of vaccine in the neonatal calves.

Injected LAK cells originating from PBMC of the dams are alloantigen to the neonatal calves. In general, T cells recognize alloantigens, which leads to the rejection of the transplanted tissues/cells. But the colostral

![Fig. 4. Changes in BHV-1 antibody titer in calves treated with LAK cells (black circles) and untreated controls (white circles). The arrows indicate vaccine inoculation.](image-url)
leukocytes of dam are adoptively transferred through the intestine and into the circulation of newborn calves (Liebler-Tenorio et al. 2002). It was reported that the collostral cells enhance neonatal immunity during the first month of life (Langel et al. 2015). Even if the LAK cells were alloantigen originating from PBMC of the dam, the injection of LAK cells might have the effect on immune activation in the newborn calves.

Finally, LAK cells from dams may have a positive influence on the development of immune system in the newborn calves. Lymphocytes from vaccinated neonatal calves had significantly higher levels of virus-specific proliferation than lymphocytes from unvaccinated calves at weaning (Ellis et al. 1996). This indicated that virus-specific memory cells persisted in calves until weaning. We suggest that LAK cells from dam lymphocyte may be effective in promoting an acquired immune function when the attenuated live BHV-1 vaccine is inoculated to neonatal calves. But it is not clear whether the LAK cells injection provides an improvement in boosting effect to the neonatal calves inoculated with live or killed virus vaccines against the other bovine virus infections, such as bovine respiratory syncytial virus, parainfluenza 3, adenovirus and bovine viral diarrhea virus. Further investigations are needed to clarify these aspects.

References


