Effects of dietary uridine 5′-monophosphate on immune responses in newborn calves

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ABSTRACT: When compared with normal milk, bovine colostrum contains a large amount of uridine 5′-monophosphate (UMP) and its derivatives. In the present study, we carried out 2 experiments to determine the effects of dietary UMP (2 g/d) on the immune status of newborn calves. In Exp. 1, newborn Holstein bull calves were fed milk replacer alone (control group) or milk replacer supplemented with UMP (UMP group) from d 4 to 10 after birth. The increase in interferon-γ concentration by peripheral blood mononuclear cells (PBMC) on d 24 tended to be greater in the UMP group than in the control group (P = 0.06). The IgA concentration of the ileal mucosa was greater in the UMP group than in the control group (P < 0.05), although there was no difference between groups in the jejunal mucosa. In Exp. 2, newborn Holstein bull calves were fed milk replacer alone (control group) or milk replacer supplemented with UMP (UMP group) from d 4 to 56 after birth. The proliferation of PBMC was greater in the UMP group than in the control group on d 14, 28, and 42 (P < 0.01). The increase in interferon-γ concentration by PBMC was greater in the UMP group than in the control group on d 28 and 42 (P < 0.05). From these results, we concluded that dietary UMP affected the immune responses of newborn calves.

Key words: interferon-γ, mucosal immunoglobulin A, peripheral blood mononuclear cell proliferation, uridine 5′-monophosphate

INTRODUCTION

Newborn calves are immediately exposed to a large number of potential pathogens at birth. Calves are immunonaïve (Barrington and Parish, 2001) and cannot produce sufficient antibodies during the first few weeks of life; instead, they acquire maternal antibodies from colostrum or milk. However, the quality and quantity of colostrum consumed are not always constant, and newborn calves occasionally do not receive sufficient maternal antibodies. Calves fed amounts of colostrum sufficient to produce serum immunoglobulin concentrations in excess of 30 mg/L did not develop diarrhea, whereas calves fed less colostrum did (McNulty et al., 1976). Calves that absorb less than adequate amounts of immunoglobulin may be susceptible to pneumonia at approximately 2 mo of age (Corbeil et al., 1984). These diseases cause great economic losses worldwide.

Bovine colostrum contains a large amount of nucleotides compared with normal milk. The composition and concentration of colostral nucleotides depend on the species. For example, ruminant colostrum predominantly contains uridine 5′-monophosphate (UMP) and its derivatives (Gil and Sanchez-Medina, 1981). In sow colostrum, UMP represents 98% of all 5′-monophosphate nucleotides (Mateo et al., 2004), whereas human colostrum contains mainly cytidine 5′-monophosphate (Duchén and Thorell, 1999). Several biological actions of dietary nucleotides, including UMP, have been reported in the immune systems of several species. For example, dietary nucleotides can upregulate the T-

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helper cell type 1 (Th1) immune response in systemic immunity (Nagafuchi et al., 1997). In addition, dietary uracil, which is the base of UMP, decreased the susceptibility to bacterial challenge in Bagg albino (BALB/c) mice (Kulkarni et al., 1986). Thus, we speculated that dietary UMP could influence the immunoreactivity of newborn calves. Our objective in the present study was to assess the effects of dietary UMP on the immune response of newborn calves.

MATERIALS AND METHODS

Newborn Holstein bull calves were treated according to the Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences (The Physiological Society of Japan, Tokyo, Japan), and the present experiment was approved by The Animal Care Committee of Tohoku University.

Animals

Exp. 1. Twelve newborn calves were fed 2 L of colostrum from their dams once after birth. The mean UMP concentration of colostrum was 220 ± 45 mg/L and there was no difference between the groups. They were then fed a commercial colostrum (Head Start, Saskatoon Colostrum Co., Saskatoon, Canada) for 3 d before beginning the experimental feeding. It contained 26.7% IgG (total CP 45%) and 0.008% UMP. This colostrum (225 g), diluted to 2 L with warm water, was given to each calf in the morning (0830 h) and afternoon (1530 h). On d 4, a total of 12 calves were assigned to 1 of 2 groups: the control group and the UMP group. The calves in the control group were fed milk replacer alone for 7 d from d 4 to 10. The calves in the UMP group were fed milk replacer supplemented with pure UMP (1 g/feeding) derived from yeast for 53 d from d 4 to 56. Milk replacer (250 g), diluted to 2 L with warm water, was given to each calf in the morning (0830 h) and afternoon (1530 h). The total amount of UMP given was 2 g/calf daily. During experimental feeding, all calves were provided a calf starter diet and timothy hay ad libitum. The calf starter (Meiji Feed Co.) was pelleted and contained corn, rye, soybean meal, rapeseed meal, minerals, and vitamins (18% CP, 2% crude fat, 0.8% Ca, 0.4% P, 10,000 IU/kg of vitamin A, 2,000 IU/kg of vitamin D, 30 IU/kg of vitamin E, and a nondetectable concentration of UMP, as-fed basis).

Sampling

Exp. 1. Blood samples (15 mL) were collected on d 3, 10, and 24 from an external jugular vein into sterile vacuum tubes containing 90 IU of heparin (Terumo Co., Tokyo, Japan). Interferon-γ (IFN-γ) assays were carried out on the days of sampling and stored at −70°C for later analysis. Body weight was measured on d 24. The calves were then anesthetized by 1.5 g/calf of thiopental sodium (Ravonal, Tanabe Pharm. Co., Osaka, Japan) and exsanguinated. Within 15 min after death, 5-cm segments of the jejunum (1 m distal to the duodenojejunal junction) and ileum (1 m proximal to the ileocecal junction) were harvested. The jejunal and ileal segments were washed with saline and mucosae were scraped out with a glass slide. Mucosa samples were frozen immediately in liquid N2 and stored at −70°C for IgA assay.

Exp. 2. Blood samples (15 mL) were collected on d 3, 14, 28, and 42 from an external jugular vein into sterile vacuum tubes, as described for Exp. 1. Peripheral blood mononuclear cell (PBMC) proliferation assays were carried out on the days of sampling. Interferon-γ assays were carried out on days of sampling and stored at −70°C for later analysis. Body weight was measured on d 56. After that, the calves were slaughtered and the weights of the liver and spleen were measured.

Analyses

Preparation of PBMC. In both experiments, PBMC were isolated from whole blood by density gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Ficoll-Paque Plus (15 mL) was added to 50-mL tubes. Whole blood (15 mL) was diluted with the same volume of balanced salt solution (5.6 mM anhydrous D-glucose, 5.0 μM CaCl2, 0.98 μM MgCl2, 5.4 μM KCl, 14.5 mM Tris, 126 mM NaCl, pH 7.6). The diluted whole blood (30 mL) was carefully layered onto 15 mL of Ficoll-Paque Plus and centrifuged at 1,460 × g for 40 min at room temperature. After centrifugation, PBMC were collected from the lymphocyte layer and erythrocytes were lysed in 0.16 M NH4Cl.

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The PBMC were then washed twice with balanced salt solution. The PBMC were counted in Türk’s solution (Merck KGaA, Darmstadt, Germany), and PBMC suspensions were prepared at a concentration of 1 × 10^6 cells/mL in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (ICN Biomedica Inc., Solon, OH), 0.05 mM 2-mercaptoethanol (Nakarai Tesque Inc., Kyoto, Japan), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco).

**Proliferation Assay for PBMC.** The PBMC were incubated in the presence or absence of cluster of differentiation 3 (CD3) antibodies (VMRD Inc., Pullman, WA), or lipopolysaccharide (LPS) (Purified LPS antibodies were diluted at a concentration of 10 μg/mL in Dulbecco’s PBS. Nissui Pharmaceutical Co., Tokyo, Japan) and adsorbed directly to wells of a flat-bottomed 96-well microtiter plate (Falcon, Becton Dickson and Company, Franklin Lakes, NJ) for 2 h at 37°C. Unbound antibodies were then washed away with Dulbecco’s PBS. Finally, 1 × 10^5 PBMC in 200 μL of RPMI-1640 medium was added to each well and incubated for 72 h under a humidified atmosphere of 5% CO₂ at 37°C. Meanwhile, 1 × 10^5 PBMC in 200 μL of RPMI-1640 medium containing 50 μg/mL of LPS (Sigma-Aldrich Inc., St. Louis, MO) or medium alone was added to wells of a flat-bottomed 96-well microtiter plate and incubated for 72 h under a humidified atmosphere of 5% CO₂ at 37°C. After incubation, cell proliferation was measured by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt assay kit (Tetra Color One, Seikagaku Co., Tokyo, Japan), which is based on the cellular conversion of a tetrazolium salt into a formazan product. Tetra Color One reagent (10 μL) was added to each well, and cells were incubated for an additional 2 h. Optical density (OD) was measured at 450 nm with a microplate reader (NalgeNunc International, Tokyo, Japan). Intraassay CV was 2.8 to 13.9%, and the limit of OD detection was 0.001.

**IFN-γ Assay and Measurement of IFN-γ.** For Exp. 1, PBMC were incubated with or without concanavalin A (ConA). The RPMI-1640 medium (100 μL) containing 6 μg/mL of ConA (Sigma-Aldrich Inc.) or medium alone was added to wells of a flat-bottomed 96-well microtiter plate. Then 1 × 10^5 PBMC in 100 μL of RPMI-1640 medium was added to each well, resulting in a final ConA concentration of 3 μg/mL. The PBMC were incubated for 72 h under a humidified atmosphere of 5% CO₂ at 37°C. After incubation, cultures were centrifuged at 440 × g for 10 min at 4°C, and supernatants were stored at −70°C until use. For Exp. 2, PBMC were incubated with or without CD3 antibodies, as described for the PBMC proliferation assay. After incubation, cultures were centrifuged at 440 × g for 10 min at 4°C, and supernatants were stored at −70°C until use. The concentration of IFN-γ in each supernatant was determined by ELISA with a commercial kit (Bovine Gamma Interferon ELISA kit, Bio-X Diagnostics, Jemelle, Belgium). The data are expressed as arbitrary units (UA) defined by this ELISA kit. Intraassay CV was 0.8 to 6.2%, and the limit of detection was 0.78 UA/mL.

**IgA Assay.** Jejunal and ileal mucosa samples were thawed, weighed, and transferred to 15-mL tubes. Mucosal tissues were homogenized (UltraTurrax T8, IKAI Werke GmbH and Co. KG, Staufen, Germany) at room temperature in 9 times their weight of Dulbecco’s PBS. Mucosa homogenates were centrifuged at 1,460 × g for 10 min at 4°C and supernatants were transferred to clean microcentrifuge tubes. The IgA concentrations were determined by using a bovine ELISA kit (E10-121, Bethyl Laboratory Inc., Montgomery, TX). Intraassay CV was 0.8 to 6.2%, and the limit of detection was 15.6 ng/mL.

**Calculations**

**PBMC Proliferation.** We defined the value obtained on d 3, before experimental feeding, as the standard. Thus, the stimulation index (SI) was calculated by using the following formula: [SI = (OD of stimulated cells/OD of unstimulated cells)/(OD of stimulated cells on d 3/OD of unstimulated cells on d 3)] in each dietary group.

**IFN-γ.** When PBMC were incubated without ConA or CD3 antibodies, the IFN-γ concentrations of culture supernatant were approximately 0 UA/mL. We therefore defined the concentrations of IFN-γ detected with ConA or CD3 antibodies as increases in IFN-γ concentration by PBMC.

**Statistics**

The data are expressed as means ± SEM. Statistical analysis of differences between groups was performed by using Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA) for unpaired Student’s t-test. Differences of P < 0.05 were considered significant.

**RESULTS**

**Exp. 1**

We used newborn calves from d 4 because the influence of UMP was expected to be more detectable. All animals of both groups were healthy, and there was no difference in BW between the groups on d 24 (47.2 ± 1.4 and 45.8 ± 1.3 kg for the control and UMP groups, respectively; P = 0.48).

No statistically different increase in IFN-γ concentration by PBMC was observed on d 3, before experimental feeding, or on d 10, the final day of experimental feeding (data not shown). However, the increase in IFN-γ concentration by PBMC on d 24 tended to be greater in the UMP group than in the control group (P = 0.06; Table 1).

The IgA concentration in the jejunal mucosa on d 24 was similar in both groups (P = 0.96). However, the
IgA concentration in the ileal mucosa was greater in the UMP group than in the control group \( (P < 0.05) \) (Table 1).

Exp. 2

Body weights and weights of the organs on d 56 are shown in Table 2. All animals of both groups were healthy, and there was no difference in the BW or weights of the organs between the groups.

Stimulation index values were not different between the groups when PBMC were stimulated by LPS (Table 3). When PBMC were stimulated by CD3 antibodies, SI values were not different between groups on d 3. However, SI values on d 14, 28, and 42 were greater in the UMP group than in the control group \( (P < 0.01, 0.01, \text{and} 0.001, \text{respectively}; \) Table 3).

The increase in IFN-\( \gamma \) concentration by PBMC on d 3, 14, 28, and 42 is shown in Table 3. No significant differences were observed in the increase in IFN-\( \gamma \) concentration by PBMC on d 3 and 14. However, the increase in IFN-\( \gamma \) concentration by PBMC was greater in the UMP group than in the control group on d 28 and 42 \( (P < 0.05 \text{and} 0.01, \text{respectively}) \).

**DISCUSSION**

Nucleotides can be synthesized endogenously and thus are not considered essential nutrients. However, animals have a continuous requirement for nucleotides, especially in systems that present a high rate of cell turnover such as the immune system and gastrointestinal tract. Nucleotides can become conditionally essential nutrients under certain circumstances (e.g., rapid growth at young ages) because endogenous synthesis may be insufficient to sustain normal function (Carver, 1999). Biological effects of dietary nucleotides have been extensively reported as described in the introduction. In many reports, mixtures of adenosine 5'-monophosphate, guanosine 5'-monophosphate, cytidine 5'-monophosphate, and UMP are used. Thus, there is little information about the individual influence of each dietary nucleotide on immunoreactivity. The colostrum of ruminants contains large amounts of UMP and its derivatives compared with normal milk, but the effects of dietary UMP on the immune responses of newborn calves have not been reported.

In the present study, we demonstrated that the increase in IFN-\( \gamma \) concentration by PBMC was greater in the UMP group than in the control group, regardless of the UMP feeding period or condition. This finding was similar to an earlier report regarding dietary nucleotides and IFN-\( \gamma \) production of spleen cells in BALB/c mice (Nagafuchi et al., 1997). Additionally, we demonstrated that the proliferation of PBMC was greater in the UMP group than in the control group. This finding was similar to an earlier report regarding nucleotide mixture and ConA-driven proliferative responses of splenocytes in BALB/c mice (Navarro et al., 1996). Therefore, we speculate that dietary UMP may improve immune responses in newborn calves and that UMP or its derivatives in cow colostrum may play an important role in immune development during early infancy. Thus, it is anticipated that dietary UMP could prevent infectious diseases and decrease the mortality rate of newborn calves.

Lipopolysaccharide stimulates B cells, whereas CD3 antibodies stimulate T cells. In the present experiment, there was no effect of UMP on the proliferation of PBMC when PBMC were stimulated by LPS. When PBMC were stimulated by CD3 antibodies, however, there was an effect of UMP on the proliferation of PBMC. These results indicate that dietary UMP promoted the proliferation of calf T cells rather than B cells. Kulkarni et al. (1984) previously reported that dietary nucleotides influence the T-cell response more than the B-cell response and that dietary nucleotides are important for the normal function of mouse T-lymphocytes. Dietary nucleotides can influence the serum antibody response by regulating the delicate balance

### Table 1

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>UMP</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-( \gamma ) increase by PBMC, 2 arbitrary units/mL</td>
<td>81 ± 21</td>
<td>214 ± 20</td>
<td>0.06</td>
</tr>
<tr>
<td>IgA concentration, 3 ( \mu g/g ) of wet tissue wt</td>
<td>Jejunal mucosa 93 ± 25</td>
<td>95 ± 15</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Ileal mucosa 74 ± 14</td>
<td>132 ± 12</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1Values are means ± SEM for 6 calves per group.
2In vitro increase in IFN-\( \gamma \) concentration by PBMC sampled on d 24 from calves fed with or without dietary uridine 5'-monophosphate (UMP) from d 4 to 10. Cell cultures were carried out in the presence of 3 μg/mL of concanavalin A for 3 d.
3Immunoglobulin A concentrations in jejunal and ileal mucosa sampled on d 24 from calves fed with or without dietary UMP from d 4 to 10.

### Table 2

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Uridine 5'-monophosphate</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, kg</td>
<td>80.3 ± 3.0</td>
<td>85.4 ± 2.5</td>
<td>0.21</td>
</tr>
<tr>
<td>Liver, g</td>
<td>1.593 ± 106</td>
<td>1.743 ± 86</td>
<td>0.30</td>
</tr>
<tr>
<td>Spleen, g</td>
<td>259 ± 24</td>
<td>240 ± 18</td>
<td>0.55</td>
</tr>
</tbody>
</table>

1Values are means ± SEM for 6 calves per group.
between Th1 and T helper cell type 2 activities (Nagafuchi et al., 1997). Furthermore, both purine and pyrimidine nucleotides are required for T-cell proliferation and survival (Quéméneur et al., 2003). The detailed mechanisms of dietary UMP action are not known, but dietary UMP may possibly affect the proliferation and differentiation of T cells either directly or indirectly, because pyrimidine-limited conditions alter the balance of Th1/T helper cell type 2 differentiation (Dimitrova et al., 2002).

In the present study, it took 2 wk for dietary UMP to affect PBMC proliferation, but it took at least 4 wk for dietary UMP to affect the increase in IFN-γ concentration by PBMC. The reasons for this difference in time course are unknown but might serve as a starting point for future mechanistic studies.

Dietary nucleotides have been reported to have many biological activities in the digestive system, including growth and development of the small intestine as well as intestinal repair after chronic diarrhea (Bueno et al., 1994). Dietary nucleotide supplementation improves some symptoms of irritable bowel syndrome (Dancey et al., 2006). However, limited information is available on the effects of dietary nucleotides on the mucosal immunity of the digestive system. We demonstrated here that dietary UMP increased the concentration of IgA in the ileum. Immunoglobulin A plays important roles in mucosal immunity; it prevents pathogenic microbes from adhering to the mucosal epithelium and can neutralize toxins and viruses. The mechanism by which dietary UMP increases the concentration of IgA is not known, but we speculate that dietary UMP could affect intrapithelial lymphocytes either directly or indirectly because dietary nucleotides can increase the proportion of the T-cell receptor γδ+ intraepithelial lymphocyte subset and also upregulate IL-7 production by intestinal epithelial cells (Nagafuchi et al., 2000). T-cell receptor γδ-deficient mice also show impaired mucosal IgA responses (Fujihashi et al., 1996). On the basis of these findings, we speculate that dietary UMP may improve the mucosal immunity of newborn calves.

In many studies, immunostimulatory effects of dietary nucleotides have been demonstrated by using a mixture of nucleotides. In the present study, we administered UMP only to newborn calves and demonstrated immunostimulation. These effects of dietary UMP were also observed previously in splenocytes from BALB/c mice (Navarro et al., 1996). In this study, we demonstrated for the first time that dietary UMP affects the immune responses of newborn calves. Specifically, we showed that dietary UMP increases the proliferation of PBMC and boosts an increase in IFN-γ concentration by PBMC, in addition to increasing the IgA concentration of the ileal mucosa. Therefore, we speculate that dietary UMP could stimulate humoral and mucosal immunity in newborn calves.

**LITERATURE CITED**


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**Table 3. Proliferation of peripheral blood mononuclear cells (PBMC) and increase in interferon-γ (IFN-γ) concentration by PBMC (Exp. 2)**

<table>
<thead>
<tr>
<th>Item</th>
<th>Proliferation of PBMC in the presence of LPS, stimulation index</th>
<th>Proliferation of PBMC in the presence of CD3 antibody, stimulation index</th>
<th>IFN-γ concentration by PBMC, arbitrary units/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>UMP</td>
<td>P-value</td>
</tr>
<tr>
<td>Day 3</td>
<td>1.00 ± 0.17</td>
<td>1.00 ± 0.16</td>
<td>1.00</td>
</tr>
<tr>
<td>Day 14</td>
<td>1.10 ± 0.25</td>
<td>1.05 ± 0.14</td>
<td>0.70</td>
</tr>
<tr>
<td>Day 28</td>
<td>1.04 ± 0.19</td>
<td>1.00 ± 0.10</td>
<td>0.63</td>
</tr>
<tr>
<td>Day 42</td>
<td>1.00 ± 0.10</td>
<td>0.92 ± 0.17</td>
<td>0.36</td>
</tr>
</tbody>
</table>

1Values are means ± SEM for 6 calves per group.
2Proliferation of PBMC from calves fed with or without dietary uridine 5′-monophosphate (UMP) from d 4 to 56. Cell cultures were carried out in the presence of 50 μg/mL of lipopolysaccharide (LPS) on d 3, 14, 28, and 42.
3Proliferation of PBMC from calves fed with or without dietary UMP from d 4 to 56. Cell cultures were carried out in microtiter plates that had adsorbed 10 μg/mL of cluster of differentiation 3 (CD3) antibodies on d 3, 14, 28, and 42.
4In vitro increase in IFN-γ concentration by PBMC from calves fed with or without dietary UMP from d 4 to 56. Cell cultures were carried out in microtiter plates that had adsorbed 10 μg/mL of CD3 antibodies on d 3, 14, 28, and 42.
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