Detection of Bovine Antibodies to the Outer Membrane of Ruminal Bacteroides succinogenes by Enzyme-Linked Immunosorbent Assay (ELISA)

Shigeru SATO, Keiji OGIMOTO, and Yutaka NAKAI

Department of Animal Microbiology, Tohoku University, Sendai 981 and 3) Department of Animal Science, Ibaraki University, Ami, Ibaraki 300-03, Japan

(Received 4 July 1989/Accepted 19 September 1989)

ABSTRACT. The enzyme-linked immunosorbent assay (ELISA) was used to detect bovine serum antibodies directed to the outer membrane antigen of a ruminal bacteria, Bacteroides succinogenes. The outer membrane antigen of B. succinogenes was highly reactive against homologous antiserum, compared with rabbit sera raised against B. ruminicola subsp. ruminicola, B. ruminicola subsp. brevis and Selenomonas ruminantium. The titers of sera from colostrum-deprived calves were negligible level, while those of sera from colostrum-fed calves were relatively high. The mean titer of sera from 10 day-old calves was significantly (p<0.01) higher than that of 40 day-old calves, and was significantly (p<0.01) lower than that of adult cattle. The mean titer of sera from dairy cows which fed high-roughage diet was higher than that of feedlot cattle which fed high-concentrate diet. These results suggest that the antibodies against the outer membrane antigen of B. succinogenes transfer to calves via the colostrum, and that the titers of cows are affected by the way of feed management. — KEY WORDS: Bacteroides succinogenes, bovine antibody, ELISA, outer membrane, rumen

Men and animals, including ruminants, contain wide ranges of circulating antibodies against gram negative bacteria, particularly against enterobacteria [1, 2]. The antibodies against indigenous microflora are considered to be natural antibodies [2], and to be produced in responses to continuous antigenic stimulation. It was suggested in ruminants that the rumen provided particularly favourable conditions for rumen bacteria to stimulate natural antibodies production [22, 23]. On the other hand, serological techniques distinguished strains of rumen bacteria such as Butyrivibrio [5], Selenomonas [6], and Bacteroides [18, 21]. We [20] demonstrated previously the species-specific antigen in the outer membrane of Bacteroides succinogenes by immunodiffusion, immunofluorescence, and immunoelectron microscopy. No previous effort has been reported to identify the antibodies in sera against purified antigen such as the outer membrane of rumen bacteria. In the present study, we established enzyme-linked immunosorbent assay (ELISA) for detecting the specific antibodies to the outer membrane antigen of B. succinogenes, and applied it to detect serum antibodies directed against the antigen in calves and adult cattle.

MATERIALS AND METHODS

Bacterial strains: Standard strains of B. succinogenes S-85 (Fibrobacter succinogenes) [14], B. ruminicola subsp. ruminicola, B. ruminicola subsp. brevis, and Selenomonas ruminantium were used. All bacterial strains were stored at −70°C in Rumen Fluid-Glucose-Cellobiose-Agar (RGCA) medium. Media and growth conditions of the bacterial species were described previously [20]. After incubation at 37°C for 48 hrs, the organisms washed three times with 0.15 M NaCl by centrifugation at 8,000 g at 4°C.
Rabbit immunization: Antisera to intact bacterial organisms were prepared in Japanese white rabbits weighing approximately 2.5 to 3.0 Kg by the procedure of Mansheim and Kasper [13]. Briefly, the animals were inoculated intravenously 6 times in the first 2 weeks. A booster injection was performed at the fourth week, and phlebotomized at the fifth week. Each inoculum dose was $5 \times 10^9$ live organisms in 0.15 M NaCl. Antisera were stored at $-20^\circ$C until use.

Agglutinating test: Agglutinating tests between organisms of B. succinogenes and the homologous or heterologous rabbit antisera were performed as described previously [15].

Preparation of the outer membrane: The outer membrane of B. succinogenes was prepared by gentle methods of heat, EDTA treatment, shearing and differential centrifugation as described previously [20]. Briefly, the pelleted organisms were suspended in a buffer solution containing 0.01 M EDTA, and incubated in a water bath at 60$^\circ$C for 30 min. The suspension was mixed in a Waring blender for 10 sec, and centrifuged at 12,000 g for 20 min. The supernatant was collected and centrifuged at 80,000 g for 2 hrs. The pellet was collected and centrifuged again at 12,000 g for 20 min and 80,000 g for 2 hrs.

ELISA: The outer membrane antigen of B. succinogenes was suspended in 0.05 M carbonate buffer (pH 9.6), and 1 $\mu$g of the antigen was added to wells of microtiter plate (Linbro/Titertek, Flow Laboratories, Inc., Verginia). The plates were incubated at 4$^\circ$C for overnight. The wells were rinsed three times with 0.01 M phosphate-buffered saline (PBS, pH 7.2) with 0.05% Tween-20 (Sigma Chemical Co., St. Louis, Mo.). Rabbit antiserum (1:1,000) or bovine serum (1:1,000 to 1:10) diluted in PBS with 0.05% Tween-20 was added to the wells, and incubated at 37$^\circ$C for 60 min. The wells were again rinsed. Horseradish peroxidase conjugated anti-rabbit immunoglobulin (Ig)G(H+L) goat serum (1:2,000) or antiserine IgG(H+L) rabbit serum (1:1,500, ICN ImmunoBionicals, Israel) diluted in PBS with 0.05% Tween-20 was added to each well as secondary antibody. The plates were incubated at 37$^\circ$C for 30 min. The wells were again rinsed, and o-phenylenediamine (Wako pure chemical Ind.), substrate of peroxidase, suspended in 0.1 M citrate buffer (pH 4.0) containing 0.3% H$_2$O$_2$ was added to the wells. The reaction was stopped at 30 min with addition of 2.5 M H$_2$SO$_4$, and the optical density (OD) at 492 nm was read in Immuno Reader (NJ-2000, Nippon Inter Med.). Control wells included antigen plus enzyme conjugate (no antiserum), antiserum plus enzyme conjugate (no antigen), or enzyme conjugate alone (no antigen or antiserum). All sera and controls were tested in duplicate. The average background of the controls was subtracted from each test serum to give the final OD reading.

Bovine sera: Serum samples were obtained from 24 calves of Japanese Black breed at colostrum-deprived 0 day, and colostrum-fed 10 days and 40 days after birth. Serum samples were obtained also from 50 adult cattle of Holstein-Fresian breed, including 25 dairy cows which fed high-roughage diet and 25 feedlot cattle which fed high-concentrate diet. All sera were stored at $-20^\circ$C until use.

Measurement of serum IgG: Concentrations of IgG in sera from calves measured by single radial immunodiffusion (SRID) kit (ICN ImmunoBionicals, Israel).

RESULTS

Detection of rabbit antibodies against the outer membrane of B. succinogenes by ELISA: The ELISA was performed with 1 $\mu$g of the outer membrane antigen of B.
succinogenes and 1:1,000 diluted homologous or heterologous rabbit antisera (Table 1). The ELISA value of the homologous antiserum was comparatively high (1.65). While the ELISA values were negligible level in the homologous antiserum absorbed with organisms of B. succinogenes, and in the heterologous antiserum to the other bacterial species such as B. ruminicola subsp. ruminicola, B. ruminicola subsp. brevis, and S. ruminantium.

Twofold serial dilutions of rabbit antisera were examined by agglutinating test against the organisms of B. succinogenes. The titer of agglutinating antibody in the homologous antiserum was high (2,560 fold). While the agglutinating antibodies of the absorbed homologous antiserum and the heterologous antisera from rabbits immunized with the other bacterial species were negative.

Detection of bovine antibodies against the outer membrane of B. succinogenes by ELISA: The ELISA values of calves and adult cattle are estimated under conditions of 1 μg antigen per well, 1:1,000 to 1:10 serum dilution, 1:1,500 enzyme-conjugated dilution and 30 min reaction (Table 2). The titers of sera from colostrum-deprived calves were negligible level, while those of sera from colostrum-fed calves were relatively high. In a serum dilution (1:100), the titers of sera from colostrum-fed calves varied from calf to calf, and those from eight of 24 calves of 40 day-old were not detectable level (Fig. 1). The mean titer of sera from 10 day-old calves was significantly (p<0.01) higher than that of 40 day-old calves. The mean titer of sera from adult cattle was significantly (p<0.01) higher than that of colostrum-fed calves. The variations of titer in sera from feedlot cattle were larger than those in dairy cows (1:100 serum dilution). The mean titer of sera from dairy cows was higher than that of feedlot cattle.

Total IgG level of calf sera: Concentrations of IgG in sera of colostrum-deprived calves were negligible level, and those of 10 day-old calves (19.5±4.5 mg/ml, Mean±SD) were higher than 40 day-old calves (15.7±5.3 mg/ml). There were positive correlation between the ELISA values (1:100 serum dilution) and total IgG level with relatively low the coefficient of correlations (0.623) (Fig. 2).

DISCUSSION

This is the first report to identify the antibodies in sera against purified antigens of rumen bacteria. The titers of sera from colostrum-deprived calves were negligible level. While the titers of the sera from colostrum-fed 10 day-old calves were significantly higher than those of 40 day-old calves. The newborn calves acquire passive immunity soon after birth by the intestinal absorption of antibodies present in the dam’s colostrum [10]. The initial increase in concentration of Igs in serum decreases during the subsequent 8–16 days by biological degradation. In the present study, we observed increase and subsequent decrease in total IgG concentration in sera from colostrum-fed calves by SRID. Sharpe et al. [22, 23] suggested that the agglutinating antibodies against rumen bacteria in the blood serum were transferred to calves via the colostrum in which antibody level was as high as serum. Although the antibody levels in colostrum were not investigated, the results in the present study suggest that the antibodies against the outer membrane of B. succinogenes transfer to calves via the colostrum. It is unknown whether the antibodies transferred inhibit colonization of B. succinogenes in the rumen of newborn calves, however, the antibodies may influence settlement of microbial flora in the rumen.

The titers of sera from adult cattle were higher than those of colostrum-fed calves, and the mean titer of sera from dairy cows
Fig. 1. ELISA values of calves and adult cattle, with use of the outer membrane antigen of *B. succinogenes* S-85 (μg/well) and bovine sera (1:100).

Fig. 2. Relationship between ELISA values (1:100 serum dilution) and total IgG level in sera from 10 day-old (●) and 40 day-old (○) calves.

Table 1. ELISA values and agglutinating titters of *B. succinogenes* S-85 against the homologous and heterologous antisera

<table>
<thead>
<tr>
<th>Rabbit antisera</th>
<th>ELISA value</th>
<th>Agglutinating titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-<em>B. succinogenes</em></td>
<td>1.65</td>
<td>2,560</td>
</tr>
<tr>
<td>anti-<em>B. succinogenes</em> absorbed by the organisms</td>
<td>0.04</td>
<td>&lt;20</td>
</tr>
<tr>
<td>anti-<em>B. ruminicola</em> subsp. <em>ruminicola</em></td>
<td>0.06</td>
<td>&lt;20</td>
</tr>
<tr>
<td>anti-<em>B. ruminicola</em> subsp. <em>brevis</em></td>
<td>0.06</td>
<td>&lt;20</td>
</tr>
<tr>
<td>anti-<em>S. ruminantium</em></td>
<td>0.14</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

Table 2. ELISA values of calves and cattle, with use of a constant outer membrane antigen of *B. succinogenes* S-85 (1μg/well) and various dilutions of sera

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Calves</th>
<th>Adult cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day-old</td>
<td>10 day-old</td>
</tr>
<tr>
<td></td>
<td>(n=24)</td>
<td>(n=24)</td>
</tr>
<tr>
<td>1:10</td>
<td></td>
<td>0.19±0.16</td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td>0.01±0.22</td>
</tr>
<tr>
<td>1:500</td>
<td></td>
<td>0.25±0.34</td>
</tr>
<tr>
<td>1:1,000</td>
<td></td>
<td>0.73±0.34</td>
</tr>
</tbody>
</table>

ELISA values are expressed as Mean±SD.

was higher than that of feedlot cattle. It is well established that *B. succinogenes* is the most important cellulolytic bacteria in the rumen under many conditions [3, 4, 8, 9]. The relationship between microflora in the rumen and various kinds of feed rations have been published [11, 12]. *B. succinogenes* is the most numerous of the bacteria in heifers fed a high proportion of hay [17]. Cellulolytic bacteria such as *B. succinogenes* is considered to be numerous in dairy cows which fed high-roughage diet. Therefore, the differences of bacterial populations in the rumen may reflect to the differences of titters between dairy cows and feedlot cattle. Previously Ogimoto *et al.* [15, 16] reported the agglutinating antibodies in bovine sera against many species of rumen...
bacteria. They observed that the immune response pattern to *B. succinogenes* was unstable in calves, and the titers against *B. succinogenes* differed from conditions of animal feeding or management such as grazing and feedlot. The antibodies against the outer membrane of *B. succinogenes* in adult cattle may be affected by the way of feed management.

The natural antibodies to *Bacteroidaceae* are widely distributed in normal humans [7, 19, 24]. Although the antibodies against indigenous bacteria such as rumen bacteria are considered to be natural antibody [22, 23], the mechanism of antibody production and immunological roles in host protection have not been revealed. Further investigations are necessary to disclose the mechanism of antibody production against rumen bacteria, and to reveal the immunological role of rumen microbial flora to immune system in calves.

REFERENCES


要約

ELISA によるウシ血清中の抗 *Bacteroides succinogenes* 外膜抗体の検出：佐藤 織・黒元敬司・中井 裕（東北大学農学部家畜衛生学教室）——ルーメン・セルロース分解菌の *B. succinogenes* から作製した細胞外膜蛋白を用いて、ELISA によりウシ血清中の抗外膜抗体を検出した。細胞外膜抗体は *B. succinogenes* の抗原とウサギ血清に特異的に反応し、*B. ruminicola* や *Selenomonas ruminantium* の抗原体ウサギ血清との間に交叉反応は認められなかった。初乳摂取前の子牛血清において、抗外膜抗体は全く検出されず、初乳摂取後には高値を示した。10日齢の子牛血清（n=24）の平均抗体価は、40日齢時に比べ有意（p< 0.01）な高値を示した。また成牛血清中の抗外膜抗体は、子牛のそれに比べ有意（p<0.01）な高値を示した。乳牛血清（n=25）の平均抗体価は、肥育牛（n=25）に比べ高値を示す傾向が認められた。抗 *B. succinogenes* 外膜抗体は、初乳を介して子牛に移行すること、およびその保有状況は、牛の飼養条件が影響していると考察された。