Localization of Bacterial Antigens in Calves Inoculated Orally with Ruminal *Bacteroides succinogenes* and *Selenomonas ruminantium*

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ABSTRACT. Localization of bacteria and bacterial materials was investigated in calves inoculated orally with live organisms of both *Bacteroides succinogenes* and *Selenomonas ruminantium* by a immunohistological method using rabbit antiserum against the outer membrane of those organisms and by a scanning electron microscope. The intact organisms of both inoculated bacterial species were detected on the rumen wall and in the lamina propria of the forestomach, and *S. ruminantium* also in the lymph nodes associated with the rumen. The bacterial materials were observed inside of macrophage-like cells in the lamina propria of the forestomach and of lymphoid cells in the lymph nodes. Number of the cells in the mesenteric lymph nodes was smaller than that of the forestomach associated lymph nodes. The results suggest that the orally inoculated bacteria may act as antigenic stimulant in the mucosa of the forestomach and associated lymph nodes of calves.—**KEY WORDS:** *Bacteroides succinogenes*, calf, forestomach, outer membrane, *Selenomonas ruminantium*.

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Development of gut-associated lymphoid tissues (GALT) in animals is stimulated by various antigens [11, 18, 25]. Antigens of the gut microbial flora stimulate the GALT, and induce local production of antibodies [4, 5]. Orally administered antigen such as microorganisms was observed to be absorbed intact from the intestinal lumen [19, 20], and stimulates systemic lymphoid tissue [2, 3, 6]. Microbial flora in the rumen of ruminants may also provide continuous antigenic stimulation for GALT. We [22] reported previously the immune response to ruminant *Bacteroides succinogenes* in calves and cattle. It is still obscure whether rumen microbial flora act as antigenic stimulant in GALT or other lymphoid tissue. Recently we [23] detected immunoglobulin (Ig)G-containing cells in the mucosal propria of the forestomach of clinically normal calves and cows. This study suggested that the development of mucosal immune responses in the forestomach might be incomplete as compared with that of the intestine.

The purpose of the present study is to evaluate antigenic stimulation of ruminal bacteria in the mucosa of the forestomach and associated lymph nodes. We investigated localization of bacteria and bacterial materials in calves inoculated orally with live organisms of both *Bacteroides succinogenes* and *Selenomonas ruminantium*.

MATERIALS AND METHODS

Bacterial inoculums: Reference strain of *B. succinogenes* S-85 and *S. ruminantium* HD-1 were used. Both cultures were maintained in Rumen Fluid-Glucose-Cellobiose-Agar (RGCA) medium at -70°C . Media and growth conditions of the bacterial species were described previously [21]. After incubation at 37°C for 48 hrs, the organisms were washed three times with 0.15 M NaCl by centrifugation at 8,000 g at 4°C . Each culture was suspended in 0.15 M NaCl (10^{10} organisms/ml).

Animals and experimental design: Clini-

cally normal 4 calves, Japanese Black breed, fed colostrum from their dams were used. The calves were inoculated orally at 15 and 16 days of age (No. 1), 2 and 3 days of age (Nos. 2, 3 and 4) with 10 ml of either bacterial suspension. Two calves (Nos. 3 and 4) were reinoculated with the same dose at 26 days of age. Autopsy was performed 1 day (No. 1) and 7 days (Nos. 2, 3 and 4) after the last inoculation. The calves fed one liter of reconstituted cow's milk twice a day before and after the inoculation, and the animals had been kept indoor separated with the dams.

Tissue samples: The mucosa of the rumen, reticulum, omasum, abomasum, jejunum, ileum, cecum, and lymph nodes associated with the forestomach as well as mesenteric lymph nodes were taken at autopsy. Tissues were fixed in 10% buffered formalin solution or in 6.25% buffered glutaraldehyde solution. The tissue blocks were embedded in paraffin, and successive thin sections (5 μ m) were made.

Outer membrane and antiserum: The outer membrane of *B. succinogenes* and *S. ruminantium*, and rabbit antiserum raised against the respective outer membrane were prepared as described previously [21]. Species-specificity of the antiserum was confirmed by immunodiffusion and indirect immunofluorescent test.

Scanning electron microscopy (SEM): Glutaraldehyde-fixed tissues (5 $\times$ 5 mm) of the rumen and cecum wall were washed twice in 0.01 M phosphate buffered saline (PBS, pH 7.2) and post-fixed by 1.0% osmium acid solution. Specimens were then dehydrated in graded alcohol and into 100% acetone. Samples were dried by liquid CO₂ at critical point, mounted on aluminum stubs with silver paint, and were examined with scanning electron microscopy (MSM-2, Hitachi, Japan).

Indirect fluorescent antibody (IFA) method: After removal of paraffin by

xylene, the sections were washed with 0.01 M PBS, and incubated with rabbit antiserum raised against the outer membrane of either *B. succinogenes* or *S. ruminantium* (1:100) for 60 min at room temperature and washed with 0.01 M PBS for 15 min. The sections were incubated in fluorescein-isothiocyanate (FITC) conjugated anti-rabbit IgG(H+L) goat serum (1:20, ICN ImmunoBiologicals, Israel) for 60 min at room temperature and washed with 0.01 M PBS for 15 min. One drop of buffered glycerol-carbonate mounting fluid (one part of 0.05 M sodium carbonate buffer, pH 9.0, and nine parts of glycerol) was placed and covered with a cover slip. Slides were examined under oil immersion with fluorescent microscope (Nikon, Japan). The number of cells which possessed the positive materials (the intact bacteria or some portions of the outer membrane of the bacteria) was counted by observation of 20 fields at random under 400 fold magnification.

RESULTS

Inoculated bacteria in the rumen and cecum: The coccoid or oval-shaped bacteria (approximately 1.0-1.5 $\times$ 1.0-2.0 μ m in size) and the rod-shaped bacteria (0.8-1.0 $\times$ 2.0-4.0 μ m in size), which may be the inoculated species, were detected on the epithelial surface of the rumen of all calves by SEM. Both bacteria attached and invaded in the desquamative epithelium (Fig. 1). Other bacteria of the different shapes were also observed on the epithelium. In the cecum, a few types of a small number of bacteria attached on the epithelial surface in all calves studied.

Bacteria and the outer membrane materials in the forestomach: A large number of intact *B. succinogenes* and *S. ruminantium* were detected by IFA on the epithelial surface of the rumen, reticulum and omasum in all calves. *B. succinogenes* was

Fig. 1. Bacteria attached on the epithelial surface of the rumen of a calf (No. 3) inoculated orally with *B. succinogenes* and *S. ruminantium*. SEM, bar=2 μ m.

Fig. 2. Bacteria and the cells which possessed bacterial materials reactive with rabbit antiserum against the outer membrane of *S. ruminantium* in the lamina propria of the omasum (No. 4). IFA method (left) and H-E stain (right).

Table 1. Numbers of the cells which possessed bacterial materials reactive with rabbit antiserum against the bacterial outer membrane in the lamina propria of the forestomach and associated lymph nodes

Calf No.	Antiserum specificity	Rumen			Reticulum			Omasum		
		Lamina	Lymph node		Lamina	Lymph node		Lamina	Lymph node	
		propria	Cortex	Medulla	propria	Cortex	Medulla	propria	Cortex	Medulla
1	<i>B. succinogenes</i>	0	0	0	0	0	0	0	nd	nd
	<i>S. ruminantium</i>	0	0	0	0	0	0	0	nd	nd
2	<i>B. succinogenes</i>	0	0	0	0	0	0	0	0	0
	<i>S. ruminantium</i>	0	0	0	0	0	0	0	0	0
3	<i>B. succinogenes</i>	0	55	30	0	50	24	3	81	32
	<i>S. ruminantium</i>	0	77	49	0	65	34	15	90	55
4	<i>B. succinogenes</i>	3	280	104	0	163	75	5	210	112
	<i>S. ruminantium</i>	6	353	199	0	207	120	7	335	148

Figures indicate number of the cells per 20 fields (400 fold magnification).
nd: not determined.

detected in the lamina propria of the rumen of one calf (No. 3), and *S. ruminantium* was detected also in the lamina propria of the rumen and associated lymph nodes of two calves (Nos. 3 and 4). In the lymph node, a few *S. ruminantium* was observed in the subcapsular sinus and follicular germinal center.

Bacterial materials-positive cells were observed by IFA in the mucosa of the forestomach and in the associated lymph nodes (Table 1). A small number of the

positive cells were observed in the lamina propria of the rumen of one calf (No. 4), and of the omasum of two among four calves (Nos. 3 and 4) (Fig. 2). These cells were morphologically macrophage. In the lymph nodes associated with the forestomach, a large number of the positive cells were detected mainly in the parafollicular area and medullary cord in two calves (Nos. 3 and 4) (Fig. 3). Distribution pattern of the cells reactive with antiserum against *B. succinogenes* and *S. ruminantium* were simi-

lar. Morphologically, these positive cells were lymphoid cell, and granular fluorescences were observed diffusely in cytoplasm. No positive cell was detected from calves inoculated twice with the bacteria.

Bacteria and the outer membrane materials in the abomasum, small intestine, cecum and mesenteric lymph nodes: Only a small number of intact *B. succinogenes* and *S. ruminantium* were detected by IFA on the epithelial surfaces of the abomasum and cecum of two calves (Nos. 3 and 4). No organism was detected in the small intestine and mesenteric lymph nodes in the same

animals. Bacterial materials-positive cells were detected in the lamina propria of the abomasum in two calves (Nos. 3 and 4), and of the small intestine and cecum in three calves (Nos. 2, 3 and 4) (Table 2). The positive cells were also detected in the mesenteric lymph nodes of two calves (Nos. 3 and 4). Distribution pattern of the cells reactive with antiserum against *B. succinogenes* and *S. ruminantium* was similar.

DISCUSSION

Many intact organisms of *B. succinogenes* and *S. ruminantium* were observed on the epithelial surface of the forestomach of all calves by IFA examination and scanning electron microscopic studies. On the epithelial surfaces of the abomasum and cecum, only a few organisms were detected, and none were observed in the small intestine. It is well known that antigens administered intraintraintestinally are absorbed and can be a stimulator for lymphoid tissue [2, 3, 6]. Several enteric pathogens have a predilection for the dome epithelial cells, i.e., some bacteria including *Salmonella* may enter the dome epithelial cells and subsequently disseminate to the host organs [14]. In other case, pathogens are closely associated with the brush border instead of entering the dome epithelial cells [7]. Furthermore, M

Fig. 3. The cells which possessed bacterial materials reactive with rabbit antiserum against the outer membrane of *S. ruminantium* in the lymph node associated with the rumen (No. 4). The cells were located in the parafollicular area. IFA method (left) and H-E stain (right).

Table 2. Numbers of the cells which possessed bacterial materials reactive with rabbit antiserum against the bacterial outer membrane in the abomasum, intestine and mesenteric lymph nodes

Calf No.	Antiserum specificity	Abomasum	Jejunum	Jejunal lymph node	Ileum	Ileal lymph node	Cecum
1	<i>B. succinogenes</i>	0	0	0	0	0	0
	<i>S. ruminantium</i>	0	0	0	0	0	0
2	<i>B. succinogenes</i>	0	5	0	8	0	7
	<i>S. ruminantium</i>	0	4	0	10	0	13
3	<i>B. succinogenes</i>	10	13	20	31	27	37
	<i>S. ruminantium</i>	16	25	32	27	35	43
4	<i>B. succinogenes</i>	6	40	48	35	66	54
	<i>S. ruminantium</i>	12	47	55	60	57	82

Figures indicate number of the cells per 20 fields (400 fold magnification).

cell, lymphoepithelial cells of Peyer's patches, has an important role in the immune responses of gnotobiotic newborn calves against intestinal antigens including enteric pathogens [25, 26]. Transport of intestinal antigen by M cells with subsequent uptake by macrophages provides antigenic stimulation to the GALT [11, 17]. On the other hand, intact organisms of *B. succinogenes* and *S. ruminantium* were observed in the lamina propria of the forestomach, and *S. ruminantium* was detected also in the lymph nodes associated with the rumen. The organisms were known to be expressed antigenicity on the rumen wall by translocation across the wall [24]. Therefore, the bacteria inoculated orally might act as antigens in the mucosa of the forestomach and associated lymph nodes of the calves. Previously Ogimoto *et al.* [15, 16] reported that immune response to *S. ruminantium* was stable in calves, and agglutinating antibody titers against *S. ruminantium* were higher than those of *B. succinogenes* in sera from adult cattle. Invasion of *S. ruminantium* to the lamina propria of the forestomach and associated lymph nodes may stimulate host immune system to produce higher titer of the agglutination antibodies.

The cells reacted with rabbit antiserum against the outer membrane of *B. succinogenes* or *S. ruminantium* were observed in the lamina propria of the forestomach and associated lymph nodes. Such reactive cells were detected also in the lamina propria of the abomasum, small intestine and cecum, and in the mesenteric lymph nodes. It is well known that the lymphoid precursor cells in Peyer's patches or other GALT of mice or rats are transformed by exposure to local available antigen, and migrate to secretory tissues through the mesenteric lymph node, the thoracic duct, and systemic circulation [8–10]. Macrophages containing rumen bacterial antigen may migrate from the lamina propria of the forestomach into the

regional lymph nodes and then may stimulate the immune system. B lymphocytes form the majority population of the germinal center, and T lymphocytes are predominant in the paracortical area of the lymph nodes [1, 12]. T lymphocyte population in the nodes proliferates when T-dependent antigens including the majority of bacteria were inoculated [13]. In the present study, many lymphoid cells reacted with rabbit antiserum against the bacterial outer membrane were observed in the lymph nodes associated with the forestomach, predominantly in the parafollicular area. The results suggest that the inoculated bacterial antigens phagocytized by macrophage may stimulate lymphocytes, especially T lymphocyte populations of the lymph nodes.

The present study suggests that rumen microbial flora may stimulate the host immune system in the forestomach or in the associated lymph nodes instead of in GALT.

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要 約

Bacteroides succinogenes および *Selenomonas ruminantium* を経口接種した子牛における菌体抗原の分布：佐藤 繁・扇元敬司・中井 裕¹⁾ (東北大学農学部家畜衛生学教室, ¹⁾ 茨城大学農学部家畜衛生学教室)——ウシの免疫システムに対するルーメン細菌の抗原刺激機構を明らかにする目的で、生後間もない子牛にルーメン由来の *Bacteroides succinogenes* および *Selenomonas ruminantium* の生菌を経口的に接種し、菌体および菌体外膜抗原の分布を検索した。 *B. succinogenes* あるいは *S. ruminantium* の菌体外膜ウサギ抗血清を用いた間接蛍光抗体 (IFA) 法および走査型電顕観察により、ルーメン粘膜における接種細菌の定着が確認された。前胃粘膜固有層において両菌体の侵入が認められ、また、第一胃付属リンパ節においては、 *S. ruminantium* 菌体が検出された。一方、IFA 法により前胃粘膜固有層のマクロファージ様細胞内および前胃付属リンパ節のリンパ球様細胞内に菌体外膜抗原が検出された。外膜抗原保有細胞は小腸と盲腸粘膜固有層および腸間膜リンパ節でも検出されたが、腸間膜リンパ節に比べ、前胃付属リンパ節において多数認められた。これらのことから、経口的に接種したルーメン細菌は、前胃粘膜および付属リンパ節において抗原刺激として作用することが示唆された。