PNEUMATOSIS CYSTOIDES INTESTINALIS IN GNOTOBIOTIC QUAILS

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Abstract: Lactose-positive bacteria facilitate the colonization in the intestine in mono-associated quails fed a lactose-containing diet, because the quail does not possess intestinal lactase. We examined the influence of the overgrowth of various human intestinal lactose-positive bacteria on pathological change of mono-associated quails fed a lactose-containing diet. The strains used in this study included Bacteroides fragilis, Bifidobacterium breve, Clostridium butyricum, Esherichia coli, Enterococcus faecalis, and Lactobacillus casei. Gas cysts, hemorrhage, and erosion were observed in the cecum of the quails mono-associated with E. coli or C. butyricum. Necrotic areas were not observed in the lesion. These lesions were diagnosed as typical pneumatosis cystoides intestinalis (PCI), and the findings were benign, neither fulminant nor fatal. Weak-gas-producing mutants (gas<sup>−</sup>) derived from C. butyricum were compared as to incidence of lesion with parent strain. The biochemical characters of the mutants were consistent with the parent strain except for less than half gas production. The incidence of lesion was significantly lower in the quails mono-associated with gas<sup>−</sup> mutant than in those of mono-associated with the parent strain. These results suggest that a large amount of gas produced by C. butyricum might be mainly related to the onset of cecal PCI. (J Toxicol Pathol 9: 131-137, 1996)

Key words: Clostridium butyricum, Gnotobiotic quail, Pneumatosis cystoides intestinalis, Necrotizing enterocolitis, Gas production

Introduction

Chickens and quails were proposed as a lactose intolerant model in human newborn, since endogenous lactase is absent<sup>1</sup>. Popoff et al.<sup>3</sup> and Bousseboua et al.<sup>3</sup> reported the production of histopathological lesions similar to necrotizing enterocolitis (NEC) in chickens or quails fed a lactose-containing diet by mono-association with Clostridium butyricum.

C. butyricum is commonly isolated from the feces of healthy human and animals<sup>4-5</sup>, and some strains have been used for probiotics in both animal and human. Several beneficial effects of these strains have been reported<sup>6-10</sup>, but on the other hand, C. butyricum has been implicated in the etiology of NEC<sup>11,12</sup>. Many bacterial species including C. butyricum have been recovered from blood, peritoneal fluid, and stool of infants with NEC, but the same organism has also been isolated from asymptomatic control patients<sup>13-15</sup>.

In this paper, in order to study the potential pathogenesis of indigenous bacteria, germfree quails were mono-associated with lactose positive strains of human intestinal indigenous bacteria and gas<sup>−</sup> mutants derived from C. butyricum.

Materials and Methods

Bacterial strains and inoculation

The following strains were used in this study: Clostridium butyricum GAI 92066, Bacteroides

Field
*fragilis* GAI0675 were stock cultures in our institute. *Bifidobacterium breve* JCM 1192, *Escherichia coli* JCM 1649, *Enterococcus faecalis* JCM 5803, and *Lactobacillus casei* JCM 1134 were obtained from Japan Collection of Microorganisms. *C. butyricum* GAI 92066 has no cytotoxic effects on HeLa cells or Vero cells, negative for DNase, protease, and neuraminidase, and does not produce any other toxins.

Each group of 14 day old germfree quail was administered orally 0.1 ml of the culture containing 10^8 viable cells per ml of one of the various strains, incubated in GAM broth (Nissui Seiyaku, Tokyo, Japan) at 37°C for 18 hours under an anaerobic condition. The colonization of mono-associated bacteria in the intestine or non-contamination of any other bacteria was checked in fresh feces twice a week.

**Derivation of gasw mutants**

*C. butyricum* GAI 92066 was grown in Todd Hewitt broth (BBL, Becton Dickinson, Cockeysville, MD, USA) in an anaerobic chamber (Model ANX-1, Hirasawa, Tokyo, Japan) containing 80% N₂, 10% H₂, 10% CO₂ for 4.5 hours at 37°C. The culture was centrifuged and washed, and then resuspended to a final concentration of ten times. After addition of 1% ethyl methanesulfonate, the cell suspension was incubated at 37°C for 16 minutes, and spread onto the Nutrient agar (Nissui Seiyaku) containing 1% lactose and 0.002% bromcresol purple. After 48-hours incubation, unchanged color of edge of colonies or dysgonic colonies was picked up and pure cultured. These mutants were examined for biochemical characters according to the method by Ueno. The gas production was examined with Durham's tube in PYL broth, consisting of trypticase 1% (BBL), yeast extract 1% (Difco Laboratories, Detroit, MI, USA), lactose 1% (Difco), cystein HCl•H₂O 0.05% (Wako Pure Chemical Industries, Tokyo, Japan) after incubation for 48 hours.

**Concentrations of acetate and butyrate, pH and bacterial counts in cecal contents**

At day 18 or 35 after administration of each bacteria, the quails were exsanguinated by incision of the carotid artery under ether anesthesia. After gross pathological examination of all organs, the cecum was removed and the concentrations of acetate and butyrate, pH, and bacterial counts of the respective cecal contents were determined. The bacterial viable count was obtained, subsequent to the incubation on the GAM-modified agar (Nissui Seiyaku) at 37°C for 48 hours in the anaerobic chamber. The concentrations of acetate and butyrate of cecal contents were measured by gas chromatography according to Ueno.

**Histopathological examination**

At necropsy, all organs including intestines were examined grossly for the presence of any lesions, which were confirmed histologically. The gastrointestinal tracts were removed and checked for intraluminal contents and mucosal lesions. Tissues were fixed in 10% buffered formalin and the intestines were rolled in Swiss-roles and embedded in paraffin, routinely. Sections were stained with hematoxylin and eosin, and examined histologically. Some sections were examined for the presence of bacteria in the tissue, using Gram stain. After the bacterial examination, the mucosal lesions in the cecum were also observed and fixed in 10% buffered formalin and the same histopathological examination was performed.
Statistical analysis

Bacterial counts, pH, and VFA concentrations were compared with Student’s t test for the difference between the parent strain and mutants of *C. butyricum*. The incidence of PCI was analyzed by Fisher’s exact probability test.

Results

Some cecal lesions were observed in 7 of 10 quails at day 18 after mono-association with *C. butyricum* GAI 92066 (Table 1). Pathological changes in other portions of the intestinal tract were not observed. Gas cysts were found in the mesentery between the cecum (Fig. 1A) and in the subserosa of the cecum, but not in the submucosa. Histologically, the gas cysts were lined by epithelioid cells, including giant cells, and granuloma with infiltration of small round cells and hyperplastic fibroblasts were found around the cysts (Fig. 1B). The quails fed a lactose-free diet and mono-associated *C. butyricum* GAI

Table 1. Cecal Change in Quails Fed a Lactose-containing Diet 18 Days after Mono-association with Several Lactose-positive Bacteria

<table>
<thead>
<tr>
<th>Strains</th>
<th>Quails with cecal lesion/total quails</th>
<th>Bacterial counts (log CFU/g)</th>
<th>pH</th>
<th>Cecal contents (mean±S.D.)^1</th>
<th>Acetate (µmol/g)</th>
<th>Butyrate (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. butyricum</em> GAI 92066</td>
<td>7/10</td>
<td>8.3±0.9</td>
<td>6.0±0.8</td>
<td>14.1±9.1</td>
<td>9.7±7.0</td>
<td></td>
</tr>
<tr>
<td><em>C. butyricum</em> GAI 92066 (fed a lactose-free diet)^2</td>
<td>0/5</td>
<td>7.9±0.4</td>
<td>6.6±0.4</td>
<td>7.8±7.0</td>
<td>1.3±0.6</td>
<td></td>
</tr>
<tr>
<td><em>B. breve</em> JCM 1192</td>
<td>0/5</td>
<td>ND</td>
<td>7.3±0.3</td>
<td>Trace</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><em>B. fragilis</em> GAI 0675</td>
<td>0/4</td>
<td>9.4±1.4</td>
<td>6.0±1.1</td>
<td>15.5±2.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> JCM 1649</td>
<td>0/4</td>
<td>10.3±0.3</td>
<td>5.5±0.5</td>
<td>26.7±7.5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> JCM 5803</td>
<td>0/5</td>
<td>8.9±0.5</td>
<td>5.8±0.4</td>
<td>5.7±1.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em> JCM 1134</td>
<td>0/5</td>
<td>7.1±0.5</td>
<td>7.7±0.2</td>
<td>1.3±0.6</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

^1: Cecal contents of a pair of cecum was analyzed separately.

^2: Lactose was replaced with corn starch.

ND: Not detected

Fig. 1. A: Numerous gas-filled cysts (arrow) between a pair of cecum. B: Gas cysts in the cecal subserosa of quail fed a lactose-containing diet, 18 days after mono-association with *C. butyricum*. Gas cysts are surrounded by epithelioid cells including giant cells. HE, B: ×100.
Table 2. Cecal Change in Quails Fed a Lactose-containing Diet 35 Days after Mono-association with Several Lactose-positive Bacteria

<table>
<thead>
<tr>
<th>Strains</th>
<th>Quails with cecal lesion/total quails</th>
<th>Bacterial counts (log CFU/g)</th>
<th>pH</th>
<th>Acetate (µmol/g)</th>
<th>Butyrate (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. butyricum</em> GAI 92066</td>
<td>4/4</td>
<td>8.4±0.8</td>
<td>5.9±1.0</td>
<td>19.7±8.6</td>
<td>7.4±8.0</td>
</tr>
<tr>
<td>(fed a lactose-free diet)</td>
<td>0/5</td>
<td>7.3±1.0</td>
<td>6.8±0.2</td>
<td>6.2±0.8</td>
<td>1.2±0.6</td>
</tr>
<tr>
<td><em>B. breve</em> JCM 1192</td>
<td>0/5</td>
<td>ND</td>
<td>7.2±0.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. fragilis</em> GAI 0675</td>
<td>0/3</td>
<td>9.7±0.8</td>
<td>5.5±0.7</td>
<td>22.1±2.7</td>
<td>ND</td>
</tr>
<tr>
<td><em>E. coli</em> JCM 1649</td>
<td>1/4</td>
<td>10.2±0.3</td>
<td>5.1±0.2</td>
<td>17.6±1.6</td>
<td>ND</td>
</tr>
<tr>
<td><em>E. faecalis</em> JCM 5803</td>
<td>0/4</td>
<td>9.2±0.7</td>
<td>6.0±0.6</td>
<td>6.4±7.3</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. casei</em> JCM 1134</td>
<td>0/4</td>
<td>7.5±0.4</td>
<td>7.9±0.4</td>
<td>0.9±0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

*: Cecal contents of a pair of cecum was analyzed separately.
*: Lactose was replaced with corn starch.
ND: Not detected

92066 showed no cecal lesion. Bacterial count, concentration of acetate and butyrate in cecal contents were higher in the *C. butyricum* group fed a lactose-diet than lactose-free diet. Butyrate was detected in only the quails mono-associated with *C. butyricum*. No particular changes could be found in the intestinal tract of quails mono-associated with other strains.

Cecal lesions were observed in all the 4 quails mono-associated with *C. butyricum* GAI 92066 and 1 of the 4 with *E. coli* at day 35 (Table 2). Histopathologically, gas cysts were found in the muscular layer (Fig. 2A), and erosion and hemorrhage were seen in the mucosa with inflammatory cell infiltration. However, there were no necrotic areas (Fig. 2B). These lesions were typical findings to diagnose PCI. Concentrations of acetate and butyrate, pH, and bacterial counts in cecal contents were similar to the values at day 18 in all groups. The concentration of acetate in the cecal contents was higher in the quails mono-associated with *C. butyricum, B. fragilis*, and *E. coli*. Although Gram staining was performed to examine for the presence of bacteria in the cysts, Gram positive substances could not be detected.

In the quails mono-associated with *B. breve*, viable microorganisms were not detected in the feces throughout this experiment, and thus *B. breve* was never colonized in the intestine. The body weight or organ weights among the groups were not different. Moreover, no deaths were encountered during this experiment.

Table 3. Cecal Change in Quails Fed a Lactose-containing Diet 35 Days after Mono-association with *Gas* Mutants of *C. butyricum*

<table>
<thead>
<tr>
<th>Strains</th>
<th>Quails with cecal lesion/total quails</th>
<th>Bacterial counts (log CFU/g)</th>
<th>pH</th>
<th>Acetate (µmol/g)</th>
<th>Butyrate (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Parent strain</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. butyricum</em> GAI 92066</td>
<td>11/10</td>
<td>8.4±0.8</td>
<td>5.9±1.0</td>
<td>19.7±8.6</td>
<td>7.4±8.0</td>
</tr>
<tr>
<td><em>Gas</em> mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>111</td>
<td>3/8</td>
<td>7.1±1.8</td>
<td>6.3±0.7</td>
<td>9.0±7.5</td>
<td>5.9±2.8</td>
</tr>
<tr>
<td>182</td>
<td>3/10*</td>
<td>8.1±1.0</td>
<td>6.3±0.5</td>
<td>8.3±6.1</td>
<td>3.6±3.5</td>
</tr>
</tbody>
</table>

*: Number of quails mono-associated with parent strain was added at day 18 and 35.
*: Cecal contents of a pair of cecum was analyzed separately.
*: Significant difference (P<0.05) from parent strain with Fisher's exact probability test.
Two \textit{gas}^w\textsuperscript{mutants} were obtained from \textit{C. butyricum} GAI 20266, and were sensitive to the bacteriophage, which had a high specificity for the parent strain\textsuperscript{17}. The biochemical characters of \textit{gas}^w mutants were consistent with the parent strain. The gas productions in PYL broth of their mutants were less than a half of parent strain.

Cecal PCI in the quails mono-associated with the \textit{gas}^w\textsuperscript{mutant} 111 or 182 was observed in 3 of 8 and 3 of 10 quails, respectively (Table 3). The incidence of PCI in quails mono-associated with \textit{gas}^w\textsuperscript{mutant} 182 was significantly lower than that in the parent strain (P<0.05). The concentration of acetic acid in the cecal contents mono-associated with the parent strain tended to be higher than that mono-associated with \textit{gas}^w\textsuperscript{mutants, but there was no significant difference.}

Discussion

In this study, gas cysts in the cecal wall and mucosal lesions were observed in the quails mono-associated with \textit{C. butyricum} or \textit{E. coli}, but, necrosis was not found and was not similar to the lesion of necrotizing enterocolitis (NEC).

In the quails at day 18 after mono–association with \textit{C. butyricum}, gas cysts appeared mainly in the mesentery between the cecum and the cecal serosa, but showed no changes in the mucosa. At day 35, gas cysts were found in the serosa and the muscular layer of the cecum, and the erosion and hemorrhage were seen in the mucosa, indicating an evidently more serious condition than those at day 18. These findings suggest that the lesions began on the serosal side and gradually proceeded to the mucosa, and the development of cecal PCI was necessary for long continuous colonization of \textit{C. butyricum} or \textit{E. coli}.

PCI is an uncommon condition of the submucosal or subserosal gas cysts in the wall of the small or large bowel\textsuperscript{18}. The clinical course of PCI is either fulminant or benign. Fulminant PCI is associated with an acute bacterial process, sepsis, and necrosis of the bowel, in which gas cysts are usually found in the submucosa and resemble acute neonatal NEC, while benign PCI can be totally asymptomatic and gas cysts occur predominantly in the subserosa\textsuperscript{18,20}.

For the last several years, this lesion has been thought to develop as follows: First, the presence of the gas–producing bacteria in the intestine; second to prior destruction of mucosa and secondary invasion by the organisms. The mechanical factors may play a role in penetration of bacteria into the intestinal wall. Several authors reported that the passage of viable bacteria from the intestinal tract through the mucosal epithelium to mesenteric lymph nodes and other organs in gnotobiotic animals as “bacterial translocation”\textsuperscript{21,22}. It is not apparent in this study whether gas cysts were formed by bacterial invasion or intraluminal pressure without bacterial invasion. In fact, bacteria were not detected in the gas cysts.

The incidence of cecal lesion was not 100% in the
quails mono-associated with *C. butyricum*, which finding was consistent with the previous report\(^1\). These results suggest that the development of these lesions might be affected by host factors as well as bacterial behavior.

Bousseboua *et al.* suggested that butyrate was the primary cause of the lesion in quails mono-associated with *C. butyricum*\(^3\). In this study, since PCI developed in the quails mono-associated with *E. coli*, which does not produce butyrate, butyrate might not be mainly responsible for the cecal lesions. Butyrate inhibits the proliferation of cells in culture\(^23\)\(24\), however, butyrate is considered as a source of energy for colonocytes\(^25\)\(26\) and exhibits a stimulatory effect on the proliferation of intestinal epithelial cells *in vivo*\(^27\)\(28\). However, under an ischemic condition in the intestine, fatty acids including butyrate might be a secondary cause of PCI, because the inhibition of fatty acid absorption from the lumen contributes to the disturbance of intestinal content neutralization\(^29\).

Since the quails exhibit a low endogenous lactase activity\(^1\) and its cecum is a blind pouch, bacteria facilitate the overgrowth in the cecum by using lactose that reaches the cecum without absorption. At this time, a large amount of gas and short chain fatty acids might be produced by *C. butyricum* or *E. coli*. Then, either the penetration of gas or the bacterial invasion might lead to the formation of gas cysts in the mesentery between the cecum.

In conclusion, the findings in this study suggested that non-pathological indigenous bacteria might be the cause of some lesion although in an uncommon condition such as germfree animal. Although the intestinal contents of newborn animals are said not to contain microorganisms for a while\(^30\), it is inadequate to directly apply our results to those in newborns.

References


