Experimental Infection of Young Chicks with Attaching and Effacing Escherichia coli

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Young chicks were inoculated with six different strains of attaching and effacing Escherichia coli isolated from the feces of calves, pigs, chicks, and humans. Colibacilli of some serotypes had colonized the cecum of chicks by 7 days after inoculation. The characteristic lesions associated with bacterial attachment were also seen on the mucosal surface of the cecum. Electron microscopy revealed numerous colibacilli closely attached to the surface membrane of enterocytes. Cell membranes formed cups and pedestals at the base of the attached bacilli. The results of this study support the conclusion that young chicks can be used as a model for the study of the lesions caused by attaching and effacing E. coli strains.

Recently, enteropathogenic Escherichia coli and enterohemorrhagic E. coli have been isolated from several animals. Some of these bacteria attach closely to enterocytes and elease the microvilli of the cells and were named attaching and effacing E. coli (AEEC) (8). Numerous studies have confirmed that AEEC strains are capable of inducing attaching and effacing lesions in animals (2-4, 8, 12-14), in tissue culture models (5-7), and in humans infected with AEEC (10). Experimental infection of 1-day-old chicks with AEEC was also attempted, but no lesions were seen (7). On the other hand, we have demonstrated that young chicks could be useful for the study of enteric infection (1, 11). Therefore, we tried to experimentally infect young chicks with several strains of AEEC and were able to demonstrate that chicks are also susceptible to infection with AEEC.

Eighty layer chicks obtained less than 12 h after hatching were used. The animals were offspring of White Leghorn chickens hatched in a commercial layer hatchery. The standard experimental diet without antimicrobial agents for young layer chicks was purchased from Kyudoh Co. Ltd. (Kumamoto, Japan).

Serotype O5:H-, O26:H11, O15:H10, O103:H-, O111:H-, and O157:H7 strains of AEEC were used as the inocula (Table 1). Strains OT-4 (O5:H-) and IY-1 (O26:H11) were originally isolated from the diarrheal feces of calves with natural infection (4, 13). Strains GP-5 (O15:H10) and ET-3 (O111:H-) were originally isolated from the diarrheal feces of a pig and a human, respectively. Strain SK-1 (O103:H-) was isolated from the intestinal contents of a chick. It was not confirmed whether this chick was diarrheic or not. Strain ATCC 43889, obtained from the American Type Culture Collection (Rockville, Md.), was used for E. coli serotype O157:H7. All strains except ATCC 43889 and ET-3 were isolated in the authors' laboratory from the ileum and/or cecum of animals showing attaching and effacing lesions. The bacteria were grown on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 24 h and then in Trypticase soy broth (BBL Microbiology Systems) and incubated in a rotary shaker (100 rpm) at 37°C for 6 h. Cell suspensions in Trypticase soy broth adjusted to concentrations of approximately 10⁹ cells per ml were used as inocula. First, 40 young chicks were divided into four groups of 10 each. The chicks in three groups were inoculated intragastrically with 1 ml of strain IY-1, SK-1, or ET-3 (Table 1). The control chicks received 1 ml of Trypticase soy broth only. A 10-ml syringe with an 18-gauge needle and plastic catheter tubing were used for inoculation of the chicks. The chicks in three other groups were inoculated with strain OT-4, GP-5, or ATCC 43889. The chicks were killed by bleeding after anesthetization with chloroform and necropsied 7 days after inoculation.

The bacterial progeny of the cecal contents were serially diluted 10-fold in saline and spread on DHL agar plates (Eiken Chemical Co. Ltd., Tokyo, Japan) before incubation at 37°C for 18 h for the isolation of E. coli. Inoculated AEEC was detected as follows. Ten lactose-positive colonies, identified as E. coli by standard biochemical tests, were selected at random from each culture of the endpoint dilution of cecal contents on a DHL agar plate. These 10 colonies were streaked on TSA plates, and their progeny were tested by slide agglutination with specific antiserum. The number of AEEC organisms in the cecal contents was computed from the proportion of the 10 colonies whose progeny gave a positive reaction in the slide agglutination test and the value of the endpoint dilution of cecal contents. The O103 and O157 antisera were produced by immunizing rabbits, and the other antisera, O5, O15, O26, and O111, were commercial products (Difco Laboratories, Detroit, Mich.). The specificity of these antisera was reported previously (9). E. coli isolated from control chicks was checked with antisera against the serotypes of all the inoculated organisms in each group.

Specimens taken from the duodenum (proximal and distal portions), jejunum-ileum (proximal, middle, and distal portions), cecum (tonsil and other portions), colorectum, liver, spleen, lung, heart, kidney, bursa of Fabricius, cerebrum, and cerebellum were fixed in 10% phosphate-buffered Formalin. These tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined for lesions by light microscopy. Immunohistochemically, the streptavidin-biotin (SAB; Scytek Laboratories, Logan, Utah) technique was applied to paraffin sections. After deparaffinization, endogenous peroxidase activity was blocked by treatment with 0.3% H₂O₂, and then the sections were washed twice in 0.05 M Tris buffer (pH 7.6). Reagents in the SAB kit were prepared according to the instructions of the manufacturer. Sections were counter-
stained with methyl green. Rabbit antiserum specific for each AEEC strain was used as the primary antibody.

The samples obtained from the middle portion of the cecum were used for scanning electron microscopy. They were fixed in 2.5% glutaraldehyde–phosphate buffer (pH 7.4) at 4°C for 2 h and washed three times for 15 min each in 0.1 M phosphate buffer. They were postfixed in 1% osmium tetroxide–phosphate buffer at 4°C for 1 h. After having been dehydrated through a graded series of ethyl alcohol-water mixtures, infiltrated with isopentyl acetate, and dried in a critical-point drying apparatus, they were fixed to the brass stub and coated with a thin layer of gold. The samples were examined with a scanning electron microscope (JEOL JSM-5400). The samples obtained from the cecum were also fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy resin (Nakarai Tesque, Kyoto, Japan). Thin sections stained with toluidine blue were examined by light microscopy to select areas for electron microscopy. Ultrathin sections were cut, stained with uranium acetate and lead citrate, and examined with a transmission electron microscope (JEOL JEM-1010).

No clinical signs in any chicks were evident 1 to 7 days postinoculation except for six chicks that died accidentally 1 day after inoculation. Neither blood nor mucus was observed in the feces, and no gross lesions were found at necropsy. In the chicks that died accidentally, there were no lesions associated with infection in the intestine at necropsy.

Numerous E. coli organisms were isolated from the cecal contents of all chicks, including the controls. The number of CFU ranged from 1.8 × 10* to 1.8 × 10^10 per g. Strains GP-5 (O157:H7), SK-1 (O103:H−), ATCC 43889 (O157:H7), ET-3 (O111:H−), and IY-1 (O26:H11) were isolated from one to six chicks in each group of 7 to 10, and the mean ± standard deviation of the log_{10} CFU ranged from 8.56 to 9.32 (Table 1). Strain GP-5 (O157:H10) was isolated from six chicks out of eight, the highest rate among all the groups. AEEC could not be isolated from birds exposed to strain OT-4 (O5:H−) or control birds.

Chicks in all groups that received AEEC showed histological changes in the cecal mucosa, although the chick exposed to strain OT-4 (O5:H−) did not have lesions (Table 1). In particular, lesions were diffusely present on the cecal mucosa of all chicks infected with strain SK-1 (O103:H−). Multifocal enterocyte degeneration and necrosis were associated with colonies of bacteria attached to the apical cell membrane in chicks infected with strain ATCC 43889 (O157:H7). The lesions were scattered foci on the mucosal surface for strains GP-5 (O157:H10), SK-1 (O103:H−), ET-3 (O111:H−), and IY-1 (O26:H11). No lesions were observed in control birds.

In the characteristic lesions, the superficial epithelial cells were irregular in shape, degenerated, and necrotic, frequently showing erosion (Fig. 1a). Brush borders were not sharply defined on the apical enterocytes at the sites of bacterial attachment. Lymphocytes and some cellular debris were present in the lamina propria and epithelial cell layer. Lymphoid follicles were often seen associated with the lesions in the lamina propria. Bacterial colonies and enterocyte changes were not observed in crypts. Numerous AEEC organisms were detected by immunohistochemistry in the cecal mucosa of chicks exposed to AEEC (Fig. 1b). AEEC could not be detected in the cecal mucosa of control birds.

The extent of bacterial colonization observed in SAB-stained sections paralleled the histological findings. Few AEEC organisms were present on the intact mucosal surface. Scanning electron microscopic examination of samples of the cecum revealed ragged lesions on the mucosal surface of infected chicks. Microvilli were lost from the surface of enterocytes, to which numerous short rod-shaped bacilli were attached (Fig. 2a). On the other hand, microvilli were markedly elongated, and the bacilli were embedded in the microvillous surface at the site where bacterial microcolonies were attached (Fig. 2b). Many intact microvilli were still present on the cell surface with no attached bacteria. Transmission electron microscopy revealed that superficial mucosal epithelial cells with numerous attached colibacilli were irregular in shape and arrangement (Fig. 3a). Numerous bacteria were found to be closely attached to the surface membranes of the epithelial cells. Some cell membranes showed cup invagination, a pedestal-like protrusion, and tentacle-like protrusions associated with the bacteria (Fig. 3b). In regions of attachment, cell microvilli were lost or disoriented, and the associated cytoskeletons were disrupted. A concentration of electron-dense material was seen beneath some adherent organisms. Numerous cytoplasmic vesicles and vacuoles were observed in the upper edge of the enterocyte beneath the attached colibacilli. Occasional enterocytes containing bacteria within cytoplasmic vacuoles were seen (Fig. 3a and b). No bacteria were seen in the lamina propria. Columnar epithelial cells were regular in arrangement, and aligned microvilli were evenly present on the surface of epithelial cell membranes in noninfected chicks.

No lesions were present in the intestine except for the cecum. There were also no changes in the liver, spleen, lung,

### Table 1. Isolation of AEEC from cecal contents and cecal lesions of young chicks necropsied 7 days after inoculation

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Serotype</th>
<th>Origin</th>
<th>No. of chicks</th>
<th>AEEC isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean log_{10} CFU ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. of chicks with lesions</td>
</tr>
<tr>
<td>IY-1</td>
<td>O26:H11</td>
<td>Calf</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>SK-1</td>
<td>O103:H−</td>
<td>Chick</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>ET-3</td>
<td>O111:H−</td>
<td>Human</td>
<td>9</td>
<td>8.78 ± 0.14</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>OT-4</td>
<td>O5:H−</td>
<td>Calf</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>GP-5</td>
<td>O157:H10</td>
<td>Pig</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>ATCC 43889</td>
<td>O157:H7</td>
<td>Human</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

* The number of AEEC organisms in cecal contents is computed from the proportion of the 10 lactose-positive colonies selected at random on the endpoint plate which were positive in the slide agglutination test, and this value is the log_{10} of the number of CFU per gram of cecal contents.

* One, two, and three chicks in the ET-3, GP-5, and ATCC 43889 groups, respectively, died accidentally 1 day after inoculation.
heart, kidney, bursa of Fabricius, cerebrum, or cerebellum of inoculated young chicks. Also, no changes were found in uninoculated chicks.

In the present study, the attaching and effacing lesions found in the cecum of infected chicks resembled those due to AEEC in humans (10), calves (4, 13), pigs (8, 14), and rabbits (2, 12) reported previously. The chicks used in this study appeared to be very susceptible to infection after inoculation with AEEC, especially with strains GP-5 (O15:H10), SK-1 (O103:H-), and ATCC 43889 (O157:H7). Chicks receiving only one oral dose of these strains became infected. It is not clear whether natural infections with enteropathogenic and enterohemorrhagic E. coli occur in poultry. However, the chick enterocytes respond to AEEC in the same manner as mammalian enterocytes, by allowing intimate attachment and forming pedestal-like protrusions. This suggests that the cellular mechanisms responsible for the attaching and effacing lesions triggered by AEEC are highly conserved among warm-blooded vertebrates.

There were some differences between chicks inoculated with the various strains of E. coli, and there was no close reciprocal relationship between the isolation of AEEC and the extent of mucosal lesions. Despite the higher rate of isolation of strain GP-5 (O15:H10), cecal lesions were quite rare. The pathogenicity of this strain for chicks was less intense than that of strains SK-1 (O103:H-) and ATCC 43889 (O157:H7). Even though strain SK-1 (O103:H-) was isolated from only 4 of 10

FIG. 1. Serial transverse sections of the cecum from a chick infected with strain SK-1 (O103:H-). (a) Superficial enterocytes are degenerated and clearly irregular in arrangement. Leukocytes (arrows) have infiltrated to the lamina propria and the epithelial layers. Hematoxylin and eosin stain. (b) SAB technique reaction for strain SK-1 (O103:H-) shows a crustlike layer of adherent bacteria on the mucosal surface.
FIG. 2. Scanning electron micrographs of the cecum from a chick infected with strain SK-1 (O103:H−). (a) The surfaces of rounded enterocytes are heavily coated by numerous colibacilli. Effacement of microvilli is evident at the sites where bacilli are detached (arrows). (b) Microvilli between attached bacterial cells and at the edge of a bacterial microcolony are clearly elongated. A mucosal surface with no associated bacteria is intact.

chicks, the highest rate of cecal lesions was found in the chicks infected with strain SK-1 (O103:H−). Strain SK-1 (O103:H−) was isolated from the intestinal contents of a 65-day-old chick and did not produce either verotoxin or enterotoxin. This chick showed poor growth and debility, although whether the feces were diarrheal was not clear. Colibacilli were confirmed by the immunohistochemical technique in the ceca of all chicks infected with this strain. This finding suggests that *E. coli* SK-1 colonized the cecum of all chicks inoculated with it. Diarrheal feces and hemorrhagic lesions were absent in the intestine,
although mucosal lesions with adherent bacteria were relatively diffuse in chicks infected with strain SK-1 in this study. Crypt abscesses and deeper tissue penetration of bacteria were observed in the cecum of piglets inoculated with \textit{E. coli} serotype O157:H7 (14). In this study, however, only multifocal attaching and effacing lesions were found in the chicks inoculated with strain ATCC 43889 (O157:H7). The failure of these strains to cause diarrhea or severe tissue damage may have been related to the extent of bacterial colonization and the lesions caused. More than two oral inoculations or suitable conditions of experimental infection may be necessary for these AEEC organisms to induce diarrhea or extensive lesions in a young chick. Cecal lesions were rare in the chicks infected with AEEC except for strain SK-1. Batt et al. (2) demonstrated that the attaching and effacing lesions in organ cultures of rabbit ileum appeared by 24 h postinoculation. On the basis of this finding, we assume that in this study, the colibacilli had already been excreted before necropsy. Examination of the early infectious stage may be necessary to detect colonization by the bacteria or production of the mucosal lesions in the intestines of young chicks. Neither lesions nor colonization by AEEC could be demonstrated in the chicks inoculated with strain OT-4 (O5:H–). Attempts to induce lesions in chicks with \textit{E. coli} S102 (O5:H+) in the early infectious stage have also failed (7). The reason for these results might be that the receptors for adhesion of AEEC of serotype O5:H– are not present on the cell membrane of the chick enterocyte.

Although useful tissue culture models have been developed (5–7), an animal model system for in vivo studies of the attaching and effacing lesions induced by AEEC also needs to be developed. It is well known that there are in vivo rabbit and piglet models for the study of AEEC pathogenicity. However, the species specificity of enteropathogenic \textit{E. coli} infection, such as in vivo infectivity of \textit{E. coli} RDEC-1, has been demonstrated (3). On the other hand, we were able to induce the characteristic attaching and effacing lesions in this model by using mammalian strains, at least the bovine, porcine, and human strains, as well as the avian strain. This finding strongly suggests that the intestinal mucosa of the chick contains specific receptors for mammalian AEEC. Newborn chicks are inexpensive and readily available from a number of commercial companies. Furthermore, young chicks are very easy to house. Even chicks less than 12 h old can take in food and water directly without the help of the parent or a human. Because of these features, we conclude that young chicks can be useful for investigation of the mechanism of intestinal damage caused by AEEC.

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REFERENCES