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Studies with Enteroaggregative *Escherichia coli* in the Gnotobiotic Piglet Gastroenteritis Model

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Two strains of enteroaggregative *Escherichia coli* of human origin fed to gnotobiotic piglets caused diarrhea or death in the majority of them. Histological examination revealed moderate hyperemia of the distal small intestine and cecum, swelling of small intestinal villi, and layers of aggregated bacteria stacked together in a mucus gel-like matrix overlying intact epithelium. These findings confirm that enteroaggregative *E. coli* strains produce distinctive intestinal lesions different from those caused by other major categories of diarrheagenic *E. coli*.

Escherichia coli strains that cause diarrhea in humans can be divided into five major discrete categories that differ in their pathogenesis, clinical presentation, epidemiology, and O:H serotypes (5). The gnotobiotic piglet model is useful for studying the pathogenesis of infection due to the different diarrheagenic *E. coli* strains (15–18). Accordingly, we have studied enteroaggregative *E. coli* (EAggEC) with this model.

EAggEC strains comprise a recently described new category of diarrheagenic *E. coli* (3, 14, 19), so-called because in the HEp-2 cell assay they exhibit an aggregative pattern of adherence to HEp-2 cells (3, 13, 14, 19). The aggregative property is dependent on the presence of a ca. 60-MDa plasmid (13, 19). In several epidemiological studies, EA ggEC strains have been found significantly more often in patients with diarrhea than in controls (2–4, 7, 20). Prospective studies in India (2, 3), Mexico (4), and Brazil (20) have incriminated EAggEC as an important agent of persistent diarrhea (duration, >14 days) in infants and young children.

EAggEC 17-2 (serotype O3:H2), which was isolated from a Chilean infant with diarrhea (7), exhibits the characteristic aggregative pattern in the HEp-2 assay (14, 19) and possesses a single 60-MDa plasmid. Strain 17-2 is the prototype EAggEC strain from which the plasmid DNA probe specific for detecting EAggEC was derived (1). Strain 221 (serotype O78:H33), isolated from a U.S. adult with traveler's diarrhea in Mexico (9), caused diarrhea when fed to volunteers (10). Strain 221 reacts with the EAggEC DNA plasmid probe but not with the enteropathogenic *E. coli* adherence factor DNA probe. A rough *E. coli* K-12 strain known to be harmless for healthy adult humans when ingested in doses as high as 10^{10} organisms (6) served as a negative control strain.

Fourteen newborn piglets from two litters were obtained by cesarean section and maintained under gnotobiotic conditions throughout the experiment (16–18). At 24 h of age, the piglets were fed 2 ml of a solution containing 1×10^{10} to 2×10^{10} viable organisms of one of three strains (Table 1). They were then observed for 3 days for diarrhea or other signs of illness.

Piglets were necropsied either when critically ill or on day 3 if they remained healthy. Samples were taken from five equally spaced sites in the small intestine (duodenum, jejunum, and upper, middle, and distal ileum [sites 1, 2, 3, 4, and 5, respectively]) and from the cecum, stomach, colon, and mesenteric lymph nodes, fixed in buffered formalin, and sectioned for light and electron microscopy (16–18). Heart blood and homogenized scrapings of the mucosal surface of the proximal, middle, and distal small intestine and the colon were serially diluted 10-fold in 0.85% saline and inoculated onto blood agar and MacConkey's agar, and the colonies were enumerated after 24 h of incubation at 37°C.

As summarized in Table 1, strain 17-2 caused death in one pair of piglets less than 18 h postchallenge, one of which had gross accumulation of fluid in its large intestine; microscopic examination was not performed because the piglets died during the night. Of the other four piglets that received strain 17-2, one pair developed diarrhea and appeared severely ill (anorexia, recumbency, and disinclination to move); no illness was observed in the third pair. Two of the six piglets who received strain 221 developed mild diarrhea (Table 1). The diarrhea fluid did not contain an appreciable number of blood cells or inflammatory cells.

TABLE 1. Clinical outcome and pathological changes in the intestinal mucosa of gnotobiotic piglets orally challenged with EAggEC or nonpathogenic *E. coli*

E. coli strain	No. of piglets inoculated	No. of piglets with:	
		Diarrhea	Abnormal mucosa[a]
EAggEC 17-2	6	4[b]	6[b]
EAggEC 221	6	2	3
Nonpathogenic K-12	2	0	0

[a] Abnormalities include edematous mucosa with congested blood vessels in the lamina propria, with a stacked-brick-like mesh of bacteria covering the enterocytes of the villus tips.

[b] Includes two piglets who died during the night with diarrheal illness.

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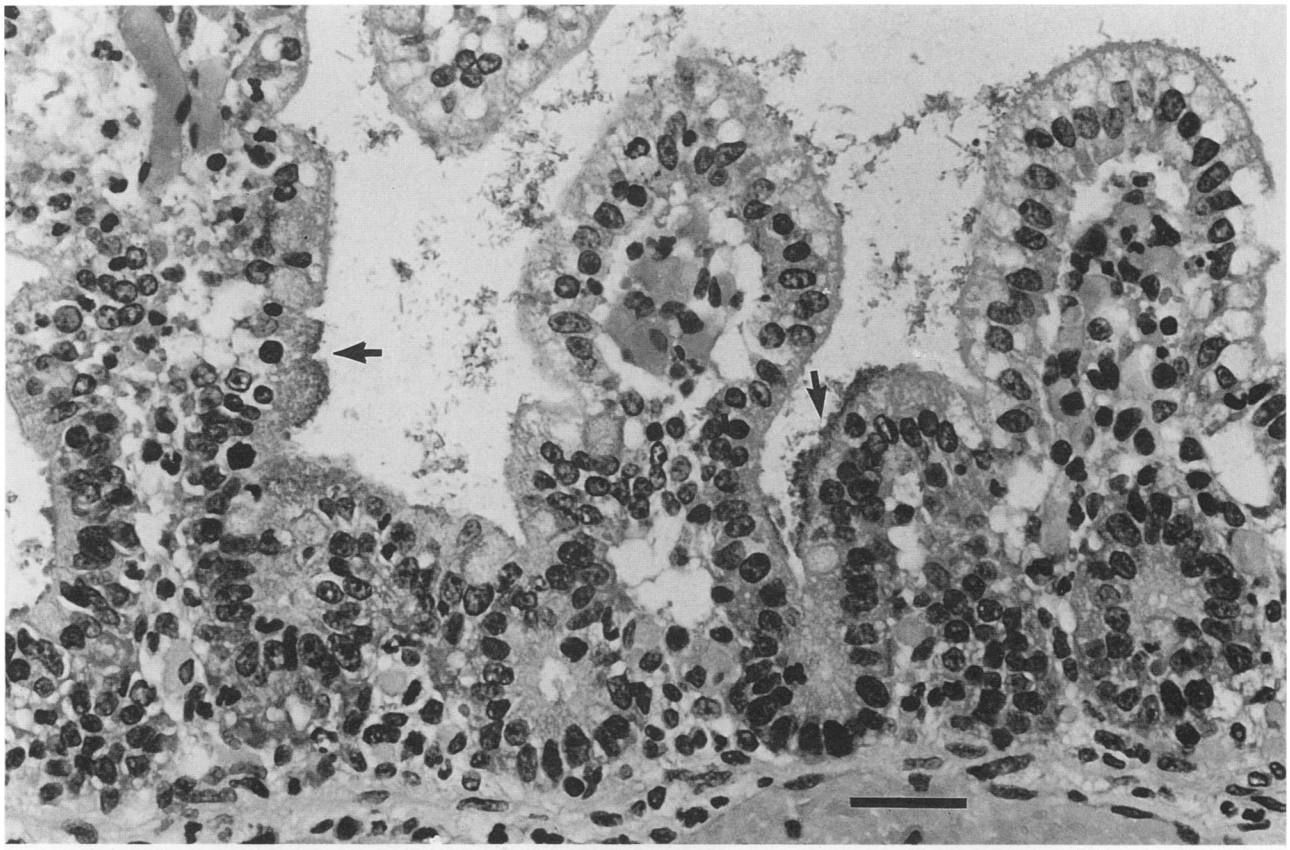

FIG. 1. Histological section of mid-ileum of a piglet euthanized 30 h after inoculation with EAggEC 17-2. Although a normal villus-to-crypt ratio is maintained (11) and there is no evidence of mucosal infiltration by inflammatory cells, the enterocytes are swollen. Moreover, in the lamina propria, there are edema and vascular congestion apparently accompanied by coagulated erythrocytes. Arrows point to thick bacterial aggregates coating the villus surfaces. Hematoxylin and eosin stain; bar = 50 μm.

With both strains 17-2 and 221, there was moderate hyperemia of the distal small intestine and cecum. In addition, there was swelling of the villi in the small intestine. Although a spectrum of clinical illness was seen in the piglets challenged with strain 17-2, all four euthanized animals exhibited essentially similar mucosal changes which were mainly in the distal half of the small intestine. The villus and crypt lengths and ratio between them remained normal, and there was no evidence of inflammation in the submucosa or the lamina propria (Fig. 1). The laminae propriae of the ilea of piglets infected with strain 17-2 exhibited edema and congestion of blood vessels and capillaries, which appeared to contain coagulated erythrocytes. One of the most striking features of the histopathology was the multiple layers of aggregated bacteria seen coating an intact surface epithelium (Fig. 2) forming a continuous bacterial sheet over a number of villus tips; this was also seen in animals challenged with strain 221. Table 2 provides a summary of the distribution in the gut of mucosal abnormalities and bacterial presence on surface epithelium. The table shows that (i) there were few

TABLE 2. Qualitative analysis of the distribution of abnormalities observed in the guts of gnotobiotic piglets infected with EAggEC or nonpathogenic *E. coli*

Strain (no. of piglets)	Severity of infection[a]						
	Small intestine site:					Large intestine	
	1	2	3	4	5	Cecum	Colon
EAggEC 17-2 (4)	−, −	−, +	+, +	++, ++	+++, +++	−, +	−, ±
EAggEC 221 (6)	−, −	−, −	−, +	−, +	++, ++	−, ±	−, ±
K-12 (control) (2)	−, −	−, −	−, −	−, −	−, −	−, ±	−, ±

[a] The first symbol of each pair indicates mucosal changes, as follows: −, no mucosal changes; +, mild changes, including swelling of villi; ++, moderate changes, including vascular congestion; +++, the above changes, with evidence of intravascular blood coagulation. The second symbol of each pair indicates presence of bacteria, as follows: −, absence of bacteria on surface epithelium; +, appearance of characteristic stacked-brick-like bacteria associated with enterocytes on a few villus tips; ++, greater degree of association; +++, continuous bacterial mesh covering most tips of villi; ±, free bacteria in the gut lumen.

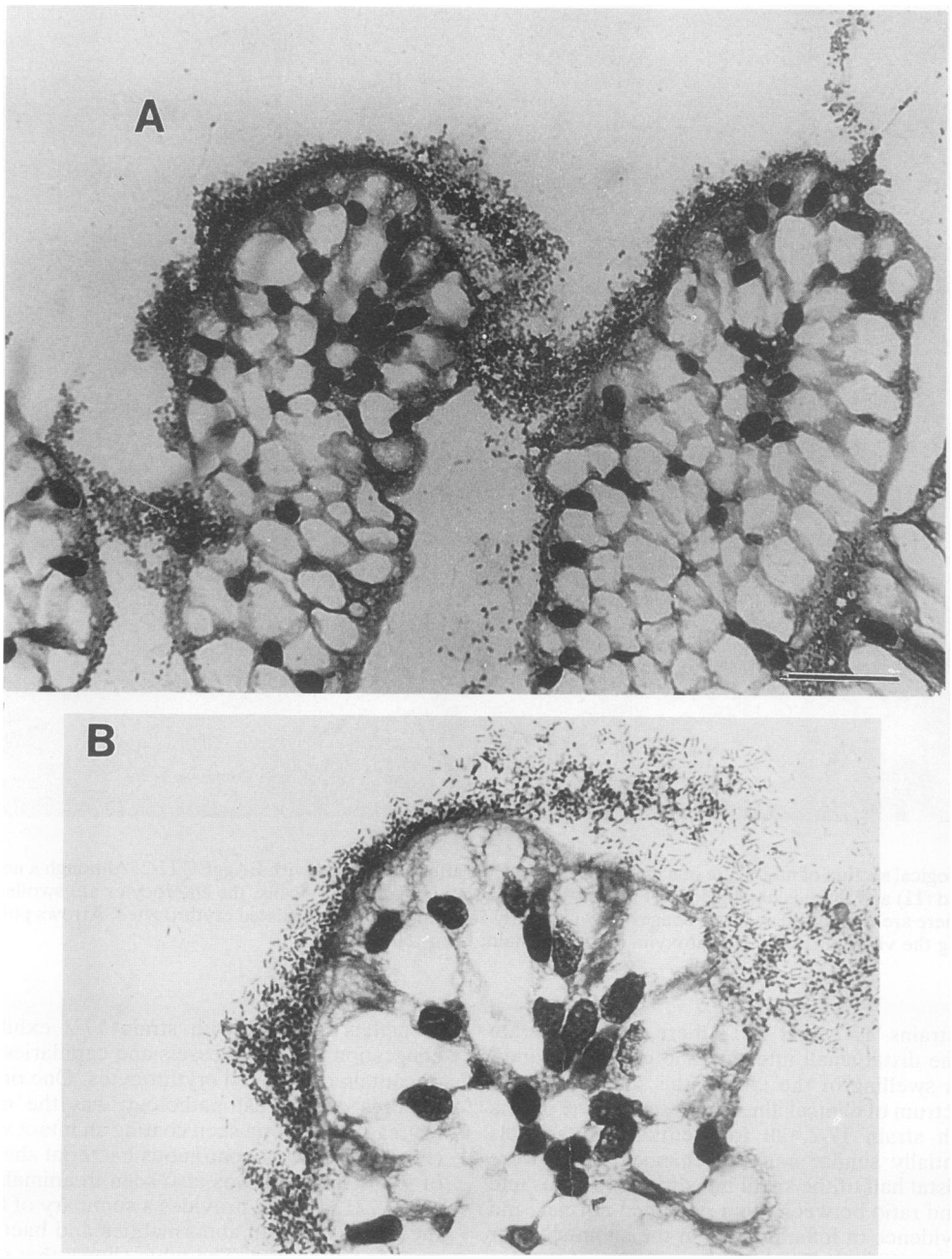

FIG. 2. (A) High-power magnification of a histological section of the mid-ileum of a piglet sacrificed 24 h after inoculation with EAggEC 17-2. Layers of aggregated bacteria are seen embedded in a mucus gel-like matrix on the surface of an otherwise normal villus. The thick bacterial aggregates are adhering intimately to the villus. Vacuolization of some enterocytes in this region of the intestine is normal in piglets at this age (12), and the cell morphology appears normal. Hematoxylin and eosin stain; bar = 50 μm. (B) A higher magnification of the villus surface shown in panel A (the tip of the left-hand villus in panel A is enlarged). The aggregative pattern of adherence to enterocytes of the villus tips closely mimics the mode of attachment of EAggEC to HEp-2 cells in tissue culture. Hematoxylin and eosin stain.

or no surface-associated bacteria or mucosal changes seen in the proximal half of the small or large intestines of all animals, (ii) there were a greater degree of bacterial association and more mucosal changes in site 4 (mid-ileum) of animals infected with strain 17-2 than in those infected with strain 221 or in controls, and (iii) there was a reasonably good correlation between the presence of bacteria on the epithelial surface and mucosal abnormalities. Electron microscopy revealed layers of bacteria stacked together and embedded in a thick mucus gel-like matrix (Fig. 3 and 4). Examination of the stomach and mesenteric lymph nodes revealed no abnormalities.

Piglets which received 10^{10} *E. coli* K-12 organisms neither developed clinical illness nor showed evidence of any mucosal alterations (Table 1).

The prechallenge coprocultures of all animals yielded no

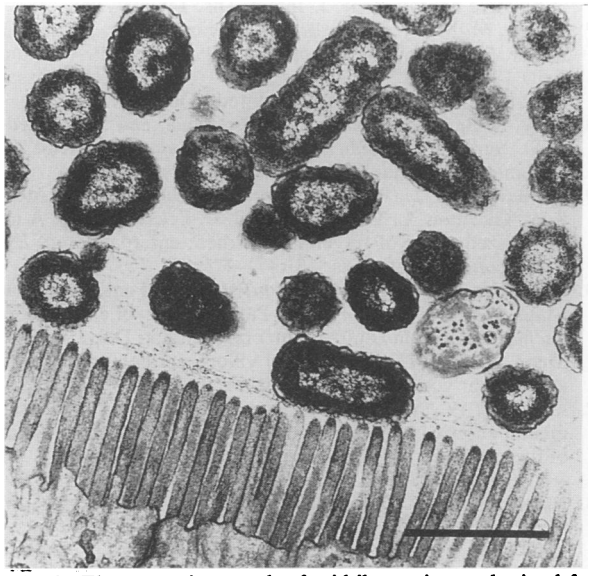

FIG. 3. Electron micrograph of mid-ileum tissue obtained from the same animal as the tissue in Fig. 2 showing a morphologically intact microvillus border with bacteria aggregating at the tips. Bar = 1 μm.

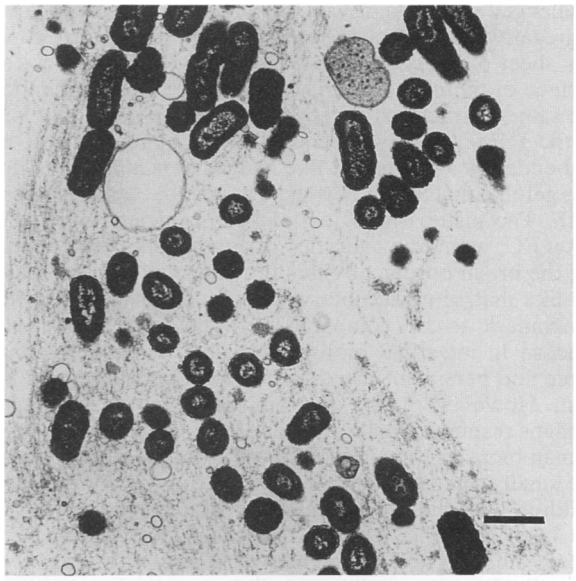

FIG. 4. Electron micrograph of mid-ileum obtained from a piglet infected with strain 17-2 showing bacterial aggregates in an intervillus space. Note that the bacteria are heavily embedded in a matrix. Bar = 1 μm.

bacteria. Postchallenge, the small intestines of piglets infected with EAggEC had *E. coli* counts ranging from 10^4 to 10^7 bacteria per g of mucosal scraping, while counts were 10^7 to 10^9/g in the large intestine (Table 3). Piglets that received the *E. coli* K-12 strain also became colonized but had approximately 1.0 to 1.5 log units fewer bacteria per g of mucosal scraping in the three of the four sites analyzed (Table 3). In each animal, the bacterium recovered from the intestine was shown to be the appropriate challenge strain. Cultures of the heart blood were negative in all animals.

The model has again provided important information by demonstrating that the features of the histopathology caused by EAggEC are distinct from any lesions that we have previously observed in piglets fed other categories of diarrheagenic *E. coli*. Both EAggEC 221 (known to cause diarrhea in volunteers) and EAggEC 17-2 (selected as the prototype EAggEC strain because it harbors only a single plasmid and thereby facilitates bacterial genetic studies) caused diarrheal illness and changes in the intestinal mucosa in a proportion of the animals. While the diarrheal response to strain 17-2 was somewhat more prominent in this study, the numbers from this initial study are too small for conclusions about their relative virulence to be drawn.

In previous studies in which EAggEC organisms were inoculated into rabbit and rat ileal loops (19), the most severe histopathological changes of the mucosa caused by the bacteria included contracted villi, denudation of villus tips, and mild hemorrhage in the submucosa. The histopathological changes observed in the piglet model were less severe than in the rabbit loops. Nevertheless, there are a number of similarities. The laminae propriae of the ilea of piglets infected with EAggEC demonstrated edema and congestion of blood vessels and capillaries. It is possible that more severe changes, such as those reported in isolated ileal loops of rats and rabbits, were not observed in the piglets because this is an open-gut model and the piglets may have been sacrificed before such changes had developed.

Rough *E. coli* K-12 does not colonize the intestine well when fed to animal models or volunteers with normal intestinal floras (6, 8), and in this sense, it is not an ideal choice as a control strain. A smooth normal-flora strain such as *E. coli* HS would have provided a more rigorous control. Nevertheless, since *E. coli* K-12 readily colonizes the intestines of gnotobiotic mice (8) and piglets (16, 18), it has been considered an acceptable and reasonable control for use in the gnotobiotic model.

Perhaps the most important observation in the piglets

TABLE 3. Quantitative bacterial counts in intestinal sites of gnotobiotic piglets inoculated with EAggEC 17-2 or 221 or nonpathogenic *E. coli* K-12 (control)

Site	No. of bacteria counted from strain		
	K-12 (2 piglets)	17-2 (4 piglets)	221 (6 piglets)
Small intestine			
Proximal	2×10^4–9×10^4[a]	4×10^4–2×10^5	2×10^4–8×10^6
Mid	3×10^4–6×10^5	8×10^4–5×10^7	7×10^4–9×10^6
Distal	2×10^5–8×10^7	3×10^6–9×10^7	2×10^5–3×10^7
Colon	7×10^6–3×10^8	4×10^8–2×10^9	6×10^7–3×10^9

[a] Counts were of approximately the same magnitude in piglets with clinical illness and those without.

challenged with EAggEC is the demonstration in vivo of the aggregative pattern of adherence to gut mucosa. A continuous sheet of EAggEC was observed covering the epithelial surface of the piglet ileum. In vivo, the bacteria manifested the same characteristic "stacked-brick" pattern that is seen in the HEp-2 cell assay and gave the appearance of being embedded in a mucus gel-like matrix. It is not clear whether this gel-like material is derived from the bacteria, the host, or both. The histopathological picture in piglets infected with EAggEC, including palisades of aggregating bacteria covering the brush border of villus tip enterocytes as well as in a gel-like matrix in the intervillus spaces, provides a helpful benchmark to be compared with the appearance of the mucosa in intestinal biopsies obtained from children with acute and persistent diarrhea due to natural EAggEC infection. However, if the predominant site of pathology in humans resembles that of piglets, for a valid comparison, the human biopsies may have to come from the distal portion of the small intestine. While results of biopsies of tissue from children with natural EAggEC intestinal infection have not yet been reported, Yamamoto et al. (21, 22) have investigated the in vitro adherence of EAggEC to native and formalin-fixed human jejunum, ileum, and colon tissues. The most avid adherence was seen with distal intestine, particularly colonic tissue, and as with the piglet model, the characteristic aggregative pattern was observed. In the model of Yamamoto et al., like in the piglet model, EAggEC adhered more readily to the tips of villi than to the sides (21, 22). The demonstration that EAggEC strains are pathogenic in the gnotobiotic piglet model paves the way for use of this model to investigate the pathogenesis of infection due to these agents.

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