

## Susceptibility of Germ-free Mice to Infectious Megaenteron

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**Abstract** Germ-free (GF)-ICR mice were shown to be less susceptible to oral inoculation with a pathogenic strain of *Escherichia coli* (*E. coli* 0115a, c: K(B)) than GF-CF#1 mice. In GF-CF#1 mice a large number of organisms were recovered from the intestinal wall from the cecum to the rectum 3 to 7 days after inoculation. Unlike those in GF-CF#1 mice, lesions in GF-ICR mice were localized in a part of the cecum and organisms were recovered only from the cecal wall and rarely from organs other than those of the alimentary tract. In both strains of mice, however, organisms were recovered in large number from the intestinal contents. Histopathology and immunofluorescence revealed organisms closely attached to the surface of the cecum, colon and rectal epithelia in GF-CF#1 mice but only in a part of the cecal epithelium in GF-ICR mice. After being in contact with conventional CF#1 mice for 21 days and then inoculated orally with the pathogenic *E. coli*, ex-GF-CF#1 mice died within 14 days with severe intestinal lesions, but ex-GF-ICR mice survived without lesions.

We previously showed that megaenteron in mice can be established in adult germ-free (GF)-CF#1 mice after oral infection with a strain of *Escherichia coli* 0115a, c: K(B), and that the infection was not fatal unless other organisms were associated with this strain of *E. coli* in the flora, suggesting the importance of the intestinal flora for producing severe desquamation of epithelial cells of the large intestine and hemorrhagic diarrhea leading to death of the host (4). Although barrier-sustained and conventional mice of the same strain were shown to have different susceptibilities, evidence of an intrinsic difference in susceptibility to the infection among mouse strains has also been obtained (5). ICR mice were found to be less susceptible to the infection than CF#1 mice while having floral organisms able to enhance the infection of GF-CF#1 mice (5).

In this study the intrinsic difference in susceptibility between GF-ICR and GF-CF#1 mice was examined and found to be related to the extent of intestinal epithelium to which the organisms adhered.

### MATERIALS AND METHODS

**Mice.** Male and female GF-CF#1 mice bred at the Institute of Medical Science, University of Tokyo, and GF-ICR mice from a commercial breeder (Japan Clea,

Kawasaki) were used at 4 to 6 weeks of age. Ex-GF mice were produced from GF mice by housing them with GF-CV-CF#1 mice (4) in an isolator. All the mice were reared in vinyl isolators with water and pellets (Oriental Yeast Co., Type NMF) sterilized in an autoclave as described previously (4).

*Organisms.* *E. coli* 0115a, c: K(B) strain Ex-30, an isolate from the cecal contents of a spontaneous case, was used. The organisms grown in nutrient broth (about  $10^8$  cells/ml) were inoculated into the stomach cavity of mice with a catheter. Details of the method are described in a previous report (4).

*Enumeration of viable organisms.* The number of viable organisms in various organs including the alimentary tract and feces of infected mice was determined by the method described previously (4). For quantitation of the organisms which were closely attached to the wall of the cecum, colon and rectum, the contents were washed three times with about 40 ml of sterilized phosphate buffered saline (PBS, pH: 7.0), and the remaining water was removed by a sterilized filter paper. A 1:10 emulsion was then made with sterilized PBS in a glass homogenizer and examined for the number of viable organisms.

*Pathology.* The severity and extension of macroscopic lesions were graded as #, +, + and - as described previously (4).

Parts of the gastrointestinal tract, liver, spleen, lung and mesenteric lymph nodes were fixed with 10% buffered formalin and embedded in paraffin and 4- to 5- $\mu\text{m}$  sections were made and stained with hematoxylin-eosin or 1.5% carbolic thionin.

*Immunofluorescence.* The direct immunofluorescence technique was applied. Antiserum to strain Ex-30 was produced by injecting a vaccine consisting of chrome-coated organisms into a rabbit, and the antibody was prepared and labeled with fluorescein-isocyanate (FITC) by the method of Kawamura (7). Frozen sections 4- to 5- $\mu\text{m}$  thick were prepared from the large intestine with a cryostat and treated with the FITC-labeled rabbit antiserum at 4 C for 18-24 hr. After being washed three times with PBS, they were observed under an ultraviolet microscope (Tiyoda Kogaku K.K., Tokyo).

## RESULTS

Fifteen GF-ICR mice were inoculated orally with strain Ex-30. All of the mice survived to 28 days without any clinical signs. Next, 18 GF-ICR mice were inoculated similarly and three mice were killed on each of days 1, 3, 5, 7, 14 and 28 and examined for macroscopic lesions as well as for the number of viable organisms in organs and intestinal contents. On days 5, 7 and 14, thickening was observed only at the tip of the cecum (Fig. 1), but no changes were seen on day 28. As shown in Fig. 2, on the day after inoculation the number of *E. coli* in the feces and large intestine reached more than  $10^8$ - $10^9$ /g, and this level was maintained for 28 days. Other parts of the alimentary tract gave the following counts during the examination period:  $10^5$ - $10^8$ /g in the stomach,  $10^3$ - $10^7$ /g in the upper part of the small intestine and  $10^4$ - $10^7$ /g in the lower part of the small intestine. Only  $10^2$ - $10^3$

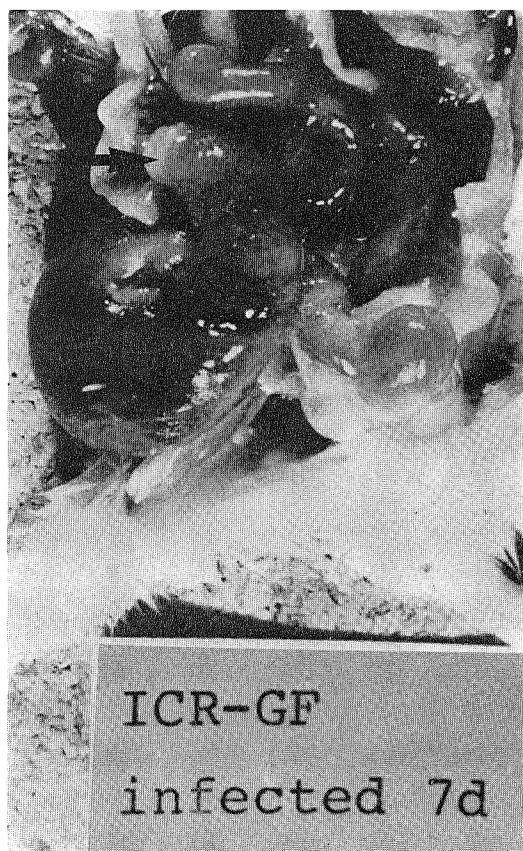


Fig. 1. Macroscopic changes in GF-ICR mice 7 days after infection with *E. coli* 0115a, c:K(B). Note thickened wall (arrow) only in a part of the cecum.

organisms/g were recovered from the liver, mesenteric lymph node, spleen and heart blood, and from only some mice on days 3 to 7, and no organisms were recovered from the kidney and lung.

Next, 12 GF-ICR mice were inoculated orally, and two mice were examined for histological changes on each day on the same schedule as in the previous experiment. Thickening was detected only in a part of the cecum. On days 5, 7 and 14, the thickened wall of the cecum showed hyperplasia of epithelial cells with some desquamation and inflammatory reaction at the lamina propria and submucosa (Fig. 3).

For bacteriological and histopathological comparison between ICR mice and CF#1 mice, 30 GF-ICR and 30 GF-CF#1 mice were inoculated orally with Ex-30 organisms, and five mice from each group were killed on days 1, 3, 5, 7, 14 and 28. Three of the mice were used for counting of viable organisms in the intestinal wall and feces, and the other two were used for histological examination.

From the feces of both ICR and CF#1 mice  $10^9$  organisms/g were recovered on days 1 to 28. From the cecal wall of ICR and CF#1 mice,  $10^8$ - $10^9$  and  $10^6$ - $10^7$

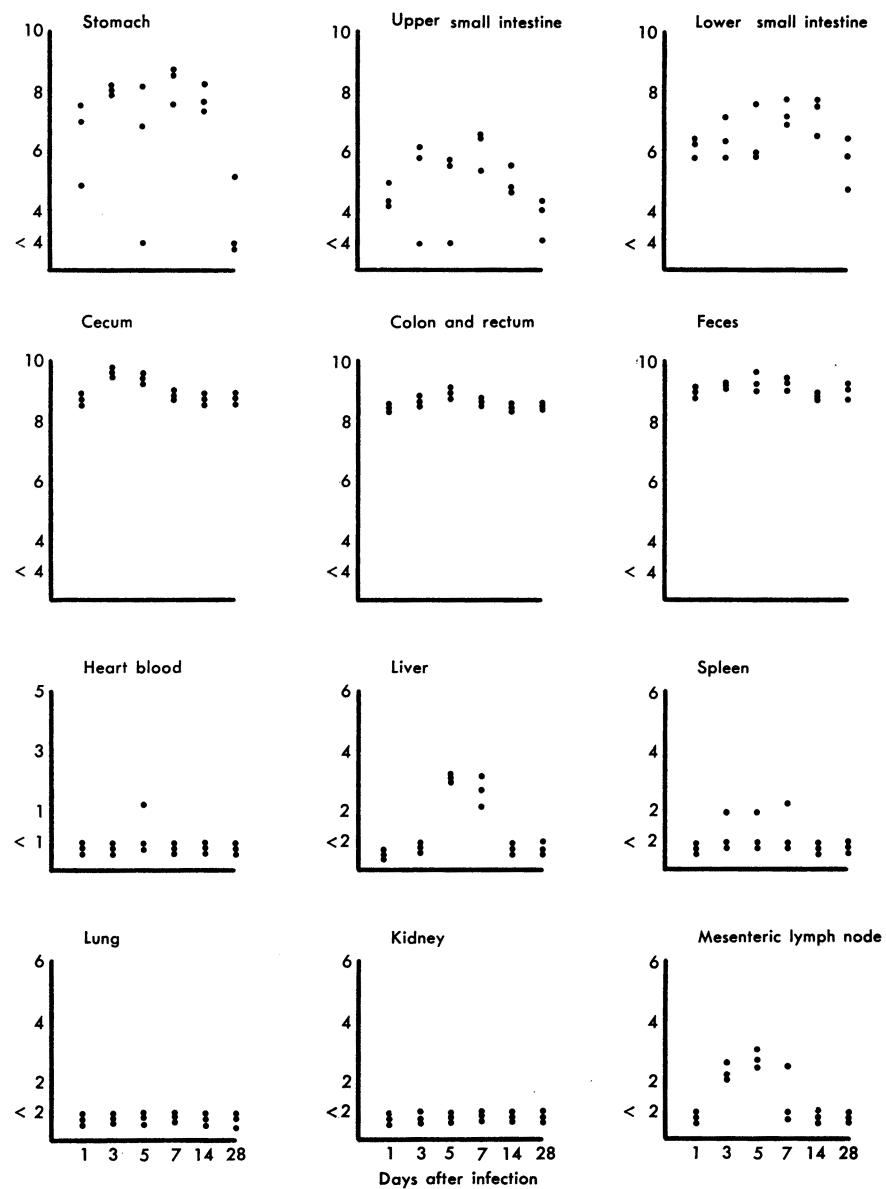


Fig. 2. Viable counts of *E. coli* 0115a, c:K(B) from various organs of GF-ICR mice after oral inoculation with  $10^7$  cells.

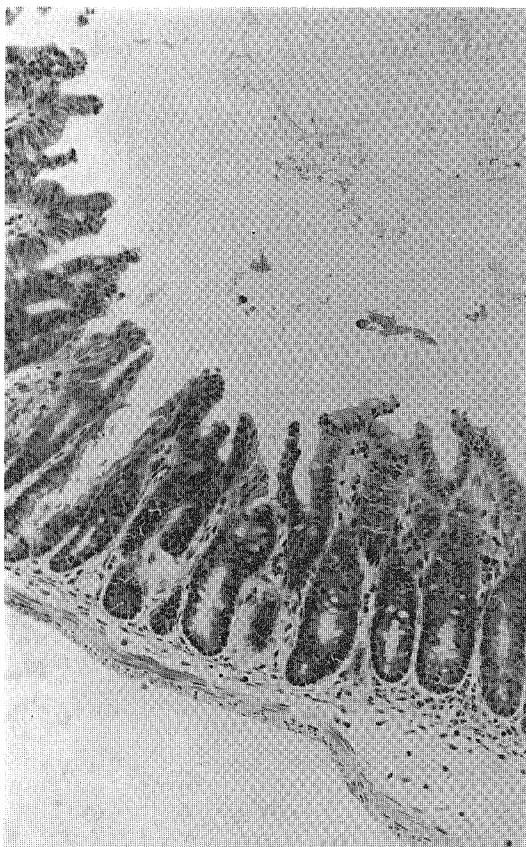


Fig. 3. Marked hyperplasia of the crypt type cells in the epithelium and edema with slight cell infiltration in the lamina propria and submucosa. The cecum of a GF-ICR mouse 7 days after infection. Hematoxylin-eosin.  $\times 100$ .

organisms/g were found on days 3 to 7 and days 14 to 28, respectively. On days 3 to 7, the number of organisms in the colon and rectal walls of CF#1 mice was similar to that of the cecum, while it was larger on day 14. In contrast, the colon and rectal wall of ICR mice contained fewer organisms throughout the experiment (Fig. 4).

By thionin staining and immunofluorescence, adherence of the pathogenic *E. coli* to epithelial cells was revealed on day 3 in the cecum, colon and rectum of CF#1 mice as well as in the cecum of ICR mice. It was seen mainly at the tip and side wall of villi on day 3 (Fig. 5), and also at the base of a villus on day 7 (Fig. 6). On days 14 and 28, no such adherence of the bacteria to the epithelium was seen. No bacteria were found in close association with the colon and rectal epithelium of ICR mice.

To determine the effect of conventionalization on susceptibility to infection, nine GF-CF#1 and 10 GF-ICR mice were cage-mated in an isolator with GF-CV-CF#1 mice which were known to be highly susceptible to pathogenic *E. coli*. After 21 days of cage-mating these ex-GF mice were inoculated orally with Ex-30 organisms. Five mice from each group were killed on day 7 and the survivors on

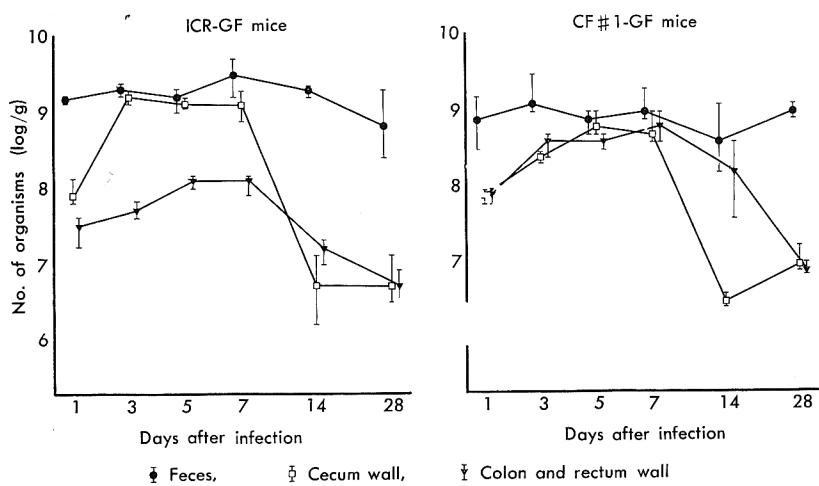


Fig. 4. Viable counts of *E. coli* 0115a, c:K(B) in feces and wall of large intestine of two infected strains of GF mice.

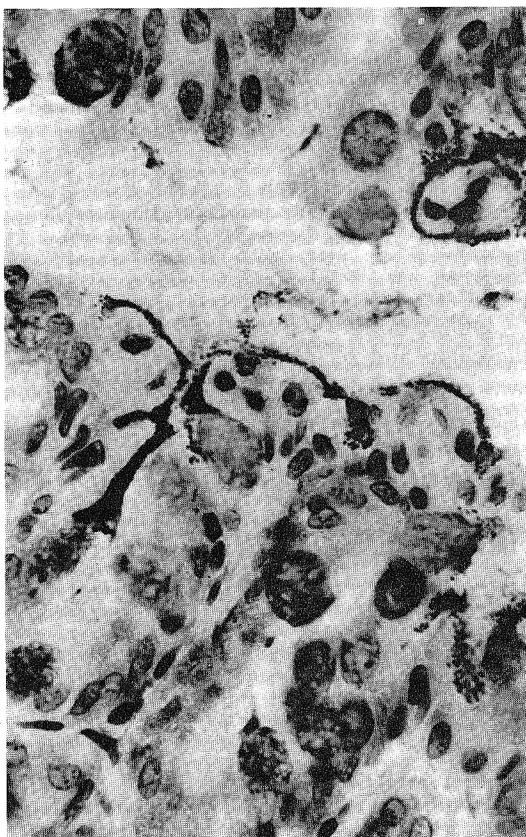


Fig. 5. Bacilli closely attached to the surface of epithelial cells. The cecum of a GF-ICR mouse 3 days after infection. Thionin stain.  $\times 400$ .

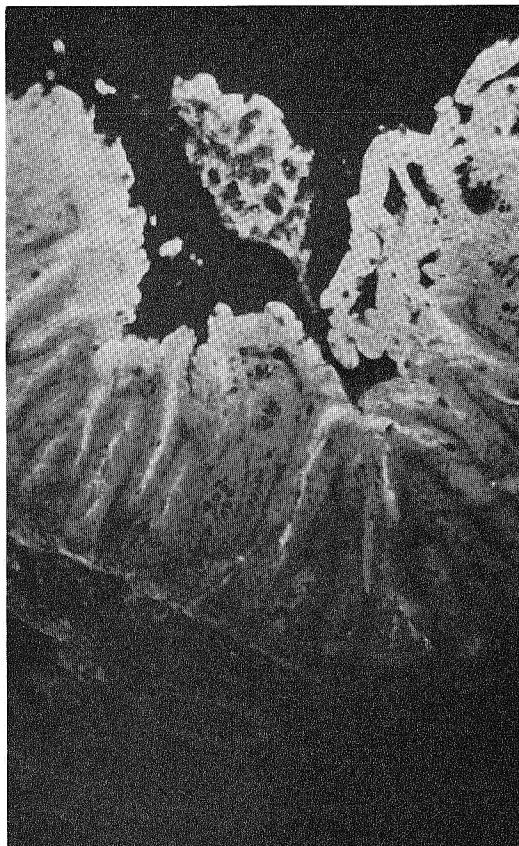


Fig. 6. The cecum of a GF-ICR mouse 7 days after infection. Bacilli attached to the surface of villi. Immunofluorescence  $\times 100$ .

day 14, and examined for viable organisms in the feces and for gross lesions. As shown in Table 1, the ex-GF-CF#1 mice had lesions of ++ or +++ grade and  $10^9$  organisms/g when examined on day 7, and all died before day 10 with severe lesions at the time of death. On the other hand, none of the ex-GF-ICR mice showed any lesions on days 7 and 14, and the numbers of organisms in the feces were  $10^8$ /g or less.

#### DISCUSSION

A difference between mouse strains in the conventional state in susceptibility to infectious megaenteron has been noted by K. Muto, National Institute of Health, Tokyo (personal communication). Our previous study with various mouse strains from barrier-sustained colonies also revealed that ICR mice were more resistant to the infection than CF#1 and DDD mice (5). On the other hand, it has been reported that a marked modification may occur depending on other organisms in

Table 1. Response of ex-GF mice to oral infection after cage-mating with GF-CV mice

Mouse strain	Days after infection <sup>a)</sup>	Mortality	Intestinal lesions <sup>b)</sup>			Number of viable organisms in intestinal contents
ex-GF-ICR	7	0/5	—	—	—	8.2±0.5 <sup>c)</sup>
	14	0/5	—	—	—	6.9±1.3
			—	—	—	
ex-GF-CF#1	7	0/5	+++	+++	+++	9.1±0.2
			++	++	++	
	14	4/4	+++	+++	+++	N.D. <sup>d)</sup>
			+++	+++	+++	

<sup>a)</sup> Mice were inoculated orally with  $10^7$  organisms.<sup>b)</sup> Grade of the lesion in individual mice examined 7 and 14 days after infection.<sup>c)</sup> Mean±S.D. (log/g).<sup>d)</sup> Not done because of death.

the intestinal flora, indicating that intrinsic factors must be studied with GF or gnotobiotic animals.

In the present study it was revealed that pathogenic *E. coli* can be established in the intestines of both GF-ICR and GF-CF#1 mice. However, lesions in GF-ICR mice were restricted to a small part of the cecum, unlike those in GF-CF#1 mice in which almost the entire length of the large intestine was involved. Such a difference in extent of intestinal lesions between GF-ICR mice and GF-CF#1 mice seems to reflect the difference in the number of organisms in the internal organs other than the intestines. There were many fewer organisms in the spleen and liver in GF-ICR mice than in GF-CF#1 mice (4). Some of the numerous organisms attached to the mucosal surface seemed to penetrate through an eroded area as discussed below. Lesions developed in the wall of the cecum, colon and rectum of GF-CF#1 mice as well as the cecum of GF-ICR mice, and organisms were recovered at a high level even after vigorous washing. In contrast, a low level of organisms was recovered from the wall of GF-ICR mice having no visible lesions, after washings.

Histologically, organisms were found to adhere closely to the mucosal surface of the cecum, colon and rectum in GF-CF#1 mice and also to that of the cecum in GF-ICR mice. In these areas, however, the organisms were seen only on the mucosal surface, not intracellularly. There have been many reports that pathogenic bacteria attach to the mucosal epithelium as the first step of infection (11, 12, 15). In megaenteron of mice also this step seems to be important for the establishment of infection. The restriction of areas susceptible to bacterial adhesion to the intestinal epithelium is probably related to the lower susceptibility of GF-ICR mice to the infection. The extent of the susceptible area might depend on the age of mice, since suckling mice have been reported to have lesions also in the small intestine after infection with pathogenic *E. coli*, while in adults they are found only in the large intestine (8). The identity of the receptors of host epithelial cells to adherence as reported in other intestinal infections and in urinary infections (1, 3, 10, 14) remains to be investigated.

Although intestinal lesions with excretion of a large number of organisms in the feces in GF-CF#1 and GF-ICR mice persisted up to the 14th day after inoculation, the number of viable organisms in the cecal wall was reduced suddenly on day 14. Histological examination at that time revealed proliferation of fibroblasts in the lamina propria and submucosa with no more organisms adhering to the epithelium. In experimental infection with *Vibrio cholerae* (2, 13) and porcine enteropathogenic *E. coli* (9), it has been reported that adherence of challenging bacteria to the intestinal epithelium did not occur in vaccinated animals, while the bacterial population in the lumen was not different from that of unvaccinated controls. In infectious megaenteron of GF-CF#1 and GF-ICR mice, serum antibody can be detected from the 14th to the 28th day after infection (unpublished observation) and might play some role in the detachment of bacteria from the epithelium.

After conventionalization infection was enhanced in ex-GF-CF#1 mice as described previously (4), but the effect was not as marked in ex-GF-ICR mice. Even after inoculation of other species of bacteria, they could not readily be colonized in ex-GF-ICR mice. Jones and Rutter reported (6) that conventional pigs but not GF pigs were protected from the colonization of pathogenic *E. coli* by their intestinal flora since the challenging organisms could not adhere to the epithelium in GF pigs. In ICR mice in which colonization of intestinal flora occurred after conventionalization, the susceptible area which is limited to a part of the cecal epithelium might have been protected from the adhesion of pathogenic *E. coli*. In ex-GF-CF#1 mice, this protective mechanism apparently did not operate effectively. The extensive susceptible area of this strain of mice, confirmed in the GF state, might provide for the pathogenic *E. coli* to have much more opportunity to adhere to the mucosal surface despite the presence of other species of bacteria, leading to aggravated infection. Although the precise mechanism of the aggravation has not been explored it is possible that the proliferation of undifferentiated crypt type epithelial cells, which is the characteristic of this infection (4), provides a vulnerable surface for synergistic attack of the potentially pathogenic *E. coli* and some other organisms.

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