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Influences of Dietary and Environmental Stress on Microbial Populations in the Murine Gastrointestinal Tract

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Aerobic and anaerobic cultural techniques and histological methods were used in a study of the effects of environmental and dietary stress on the indigenous microbiota of the gastrointestinal tract of mice. Mice previously inoculated with *Salmonella typhimurium* were examined in a similar manner. Three strains of mice (CD-1, Ha/ICr, and C57BL) were used. Control animals previously inoculated with *S. typhimurium* had low population levels of *Salmonella* bacteria in the small and large bowel. Mice previously inoculated with *Salmonella* and then deprived of food, water, and bedding for 48 h harbored high population levels of these bacteria in their small and large bowels. Coliforms increased in numbers in the large bowel of stressed mice inoculated with *Salmonella* and in the jejunum-ileum and cecum of stressed mice not previously inoculated with *Salmonella*. Control mice had high population levels of lactobacilli inhabiting the keratinized squamous epithelium of the stomach. Stressed mice showed dramatic reductions in these populations of lactobacilli. Populations of fusiform-shaped bacteria associated with the mucosal epithelium of the cecum and colon in control mice were reduced in stressed mice as determined by microscope examination of histological sections. Total anaerobic counts were similar, however, in both stressed and control animals. Environmental and dietary stress markedly alter the gastrointestinal microbiota in mice. Therefore, such stressful conditions profoundly affect the factors that regulate the localization and population levels of microorganisms in the stomach and intestines.

Adverse dietary and environmental conditions appear to precipitate overt disease in domestic animals infected with members of the genus *Salmonella* (15, 16). This situation can be mimicked in an experimental mouse model. Mice previously inoculated by the intranasal route with *S. typhimurium* are deprived subsequently of food, water, and bedding. Prior to such a stress period, low population levels of *Salmonella* are present in the gastrointestinal tract. After being stressed, the animals harbor large populations of the pathogen in their small and large bowels (27).

Food, water, and bedding deprivation is a complex stress and undoubtedly influences many host factors. The indigenous microbiota of the gastrointestinal tract may be one such factor. Experimental evidence exists suggesting that the indigenous microbiota of the gastrointestinal tract can interfere either directly or indirectly with the establishment of some pathogens in this region of the host. An example of direct interference in the large bowel may be the toxicity of volatile fatty acids for *Salmonella* and *Shigella* under conditions of low Eh and pH (11, 12). An example of indirect interference may be the effect on pathogens of stimulation of bowel motility by the indigenous microbiota. The motility of the gastrointestinal tract in germfree mice is much slower than in conventional animals. *S. typhimurium* reaches higher population levels in the small bowel of germfree mice than in the bowels of conventional animals (1, 2). Under normal conditions, bacterial pathogens may be propelled so rapidly through the intestine of conventional mice that they are unable to become established.

Such observations indicate strongly that alterations to the indigenous microbiota caused by dietary or environmental stress may have profound influences on the establishment of a pathogen in the intestinal tract. Both direct and indirect interference between the indigenous microbiota and bacterial pathogens may well be mediated by the same factors that regulate the population levels and localization of the various types of indigenous microorganisms in the gastrointestinal ecosystem (18). We are investigat-

ing the change in the indigenous microbiota in the gastrointestinal tracts of mice subjected to dietary and environmental stress. This investigation provides clues as to factors that regulate the indigenous microbiota. In the long run, we believe that the definition of such factors will help explain how indigenous microbes interfere with establishment in the bowel of pathogens such as *S. typhimurium*.

MATERIALS AND METHODS

Mice. CD-1 (Charles River, Wilmington, Mass.) and ARS Ha/ICr (A. R. Gibco, Madison, Wis.) specific-pathogen-free males were bred in our own colony. C57BL/6St Crl (Charles River, Wilmington, Mass.) female mice were purchased directly from the suppliers. Although described as Caesarian obtained-barrier sustained, these latter mice were found to carry a heavy nematode burden in the colon. The animals were housed in plastic cages with Isocaps (Isocage, Carworth, New City, N.Y.) containing Ab-Sorb Dri (Allied Mills, Chicago, Ill.) and given Lab-Blox (Allied Mills) and acidified water (21) ad libitum.

Experimental procedures. Groups of mice, 5 to 6 weeks old, were divided into two subgroups of equal numbers. The animals in one subgroup, the controls, were maintained under the conditions described above. The remaining mice were deprived of food, water, and bedding (stressed) for 48 h. In other experiments, mice were inoculated intranasally (26) with approximately 10^3 *S. typhimurium* strain LT2 (L. Rothfield, Connecticut Health Center, Farmington, Conn.). Seven days later, half of the inoculated mice were deprived of food, water, and bedding for 48 h.

Preparation of gastrointestinal specimens. Control animals and mice subjected to food, water, and bedding deprivation for 48 h were killed with chloroform. At autopsy, the stomach, cecum, colon, and a combined specimen of jejunum and ileum were removed from each animal and used for microbiological culturing or histological examination.

Aerobic culture techniques. Portions (0.5 g) of the specimens described above were homogenized with Teflon grinders in 4.5 ml of sterile brain heart infusion broth (Difco) and diluted in the same medium in 10-fold steps. Calibrated loopfuls of each dilution were spread onto Tergitol-7-triphenyl-tetrazolium medium (23) selective for coliform bacteria, methylene blue medium (23) selective for enterococci, brilliant green agar (BBL) selective for *Salmonella*, medium 10A (22) selective for *Lactobacillus*, and Sabouraud dextrose agar (Difco) containing 4,000 U of penicillin and 4 mg of streptomycin per 100 ml, selective for yeasts. The remaining homogenates were added to 10-ml volumes of tetrathionate enrichment broth (BBL). All inoculated media were incubated aerobically at 37 C for 24 h, with the exceptions of medium 10A, which was incubated in a candle jar for 48 h, and Sabouraud dextrose agar, which was left at 37 C for 72 h. Tetrathionate broths were subcultured onto brillant green agar. Estimates of the bacterial populations contained in each of the specimens were made from counts of colonies appearing on the media (23).

Anaerobic culture techniques. Samples (0.5 g each) of cecum and colon were homogenized separately with a Teflon grinder in 4.5 ml of prereduced Sweet E broth (7; gelatin and agar omitted, 10% rumen fluid) under a stream of an oxygen-free gas mixture (argon, 97%; carbon dioxide, 3%). The homogenates were diluted in the same medium in 10-fold steps by using 10-μliter pipettes (Corning Glass Works, Corning, N.Y.) and cultured in roll tubes containing prereduced brain heart infusion agar-supplemented or Sweet E agar (see above, plus 2.5% agar) (7). All manipulations were carried out with a V.P.I. anaerobic culture system (Bellco Glass, Vineland, N.J.) under oxygen-free argon-carbon dioxide. The culture tubes were incubated at 37 C for 7 days. Estimates of the bacterial populations per gram of specimen were made from colony counts.

Histological methods. Portions of specimens from the murine gastrointestinal tract were frozen with contents intact in 2% methyl cellulose in saline. The tissues were sectioned at 4 μm on a microtome cryostat and fixed onto slides in absolute methanol. Sections were stained by either a tissue Gram stain or hematoxylin and eosin.

RESULTS

Mice. CD-1 and Ha/ICr mice, subjected to food, water, and bedding deprivation (stress) for 48 h, weighed from 5 to 8 g less at autopsy than did controls. The inbred C57BL mice were much smaller than the CD-1 and Ha/ICr animals. The difference in weight between stressed and control C57BL mice was only approximately 4 g.

Aerobic gastrointestinal microflora. The aerobic microflora of control mice of the three strains varied in complexity but included at similar population levels in all animals lactobacilli, enterococci, and at least one member of the coliform group of bacteria. In stressed mice, the microflora of the various regions of the gastrointestinal tract differed markedly from that of the controls. The results obtained from Ha/ICr mice are given in Table 1. Changes in the population levels of lactobacilli in the stomach and coliforms in the jejunum-ileum and cecum were consistently observed in all three mouse strains (Table 2). Although stressed Ha/ICr mice did not show an increase in the numbers of coliform bacteria and enterococci in the colon (Table 1), the other two mouse strains showed increased populations (1 to 2 logs) of these microorganisms in this organ. The populations of enterococci were also increased by the same order in the jejunum-ileum and cecum of these strains. The inbred C57BL mice were the only ones of the animals of the three strains harboring yeasts in all regions of their gastrointestinal canals. The population levels of these organisms fell dramatically from control levels

TABLE 1. *Aerobic gastrointestinal microflora of Ha/ICr mice stressed with food, water, and bedding deprivation*

Specimen	Group	Log_{10} viable count/g				
		Lactobacillus	*Salmonella*	*E. coli*	Enterococci	Yeasts
Stomach	Stress[a]	5 (5-6)[b]	N[c]	5 (4-6)	4 (4-5)	N
	Control	9 (8-9)	N	4 (3-7)	4 (N-6)	N
Jejunum + ileum	Stress	7 (6-8)	N	7 (6-8)	6 (5-6)	N
	Control	8 (8-9)	N	4 (3-5)	5 (4-6)	N
Cecum	Stress	8 (8-9)	N	8 (7-9)	7 (5-7)	N
	Control	9 (8-9)	N	5 (5-6)	6 (5-6)	N
Colon	Stress	7 (5-8)	N	6 (5-8)	5 (4-6)	N
	Control	8 (8-9)	N	6 (5-6)	6 (4-6)	N

[a] Deprived of food, water, and bedding for 48 h.
[b] Median and range of population estimates; five mice per group.
[c] Cultures negative.

TABLE 2. *Summary of changes from normal in the aerobic gastrointestinal microflora of mice stressed with food, water, and bedding deprivation for 48 h*

Specimen	Consistent observation in stressed mice of all strains	Microorganism(s) involved
Stomach	Decrease in lactobacilli	*Lactobacillus*
Jejunum + ileum	Increase in coliforms	NLF[a] (CD-1)[b] *E. coli* (Ha/ICr) *Klebsiella* (C57BL)
Cecum	Increase in coliforms	NLF (CD-1) *E. coli* (Ha/ICr) *Klebsiella* (C57BL)
Colon	No consistent findings	

[a] A non-lactose-fermenting variant of *E. coli*.
[b] Mouse strain in parentheses.

(2 to 4 logs) throughout the tracts of stressed animals.

Mice previously inoculated with *S. typhimurium* and then stressed had increased *Salmonella* populations in the small and large bowel compared with control animals. Changes in the population levels of indigenous microorganisms in the gastrointestinal tract of *Salmonella*-inoculated mice subjected to stress were similar to those in noninfected animals. The lactobacillus population of the stomach was reduced from normal, and coliform population levels increased over control values in the large bowel of stressed mice. In contrast to noninfected animals, coliform populations in the jejunum-ileum of *Salmonella*-infected mice remained at about control levels (Tables 3 and 4).

Anaerobic microflora of the cecum and

TABLE 3. *Aerobic gastrointestinal microflora of Ha/ICr mice inoculated with S. typhimurium strain LT2 and then stressed with food, water, and bedding deprivation*

Specimen	Group	Log_{10} viable count/g				
		Lactobacillus	*Salmonella*	*E. coli*	Enterococci	Yeasts
Stomach	Stress[a]	4 (N-7)[b]	E[c] (E-4)	4 (N-7)	4 (N-7)	N
	Control	8 (7-9)	E (N-3)	5 (3-5)	3 (N-5)	N
Jejunum + ileum	Stress	5 (N-7)	5 (E-6)	6 (4-9)	6 (N-7)	N
	Control	8 (7-8)	E (N-E)	5 (4-6)	5 (3-6)	N
Cecum	Stress	8 (7-8)	5 (E-7)	10 (8-10)	8 (7-9)	N
	Control	8	E (E-4)	6 (5-7)	5 (5-7)	N
Colon	Stress	7 (7-8)	4 (E-7)	9 (7-9)	8 (7-8)	N
	Control	8	E (N-4)	6 (5-7)	5 (5-7)	N

[a] Deprived of food, water, and bedding for 48 h.
[b] Median and range of population estimates; five mice per group; N, culture negative.
[c] Only enrichment culture positive.

colon. Similar population levels of anaerobes were obtained from mice of all three strains. No consistent differences were detected between the estimates for stressed and control animals. The estimates for CD-1 mice are shown in Table 5.

Histology. As judged from microscope examination of Gram-stained histological sections, the distribution of the indigenous microbiota differed markedly from normal in the tracts of stressed mice of all the strains. In the stomach, the thick layers of gram-positive rods (lactobacilli) inhabiting the keratinized squamous epithelium of control mice were absent in stressed animals. Only a few scattered bacteria were associated with this tissue (Fig. 1a, b). The yeast layer present on the secreting epithelium of the stomachs of C57BL control mice was absent in stressed animals (Fig. 1c, d). The few scattered yeast cells remaining were elongated in shape in comparison to the spherical to ellipsoidal cells seen in control animals (Fig. 1e, f).

In sections of jejunum and ileum, numerous bacterial cells could be seen in approximately half of the animals in each stressed group (Fig. 2a, b). As detected by cultural techniques, the population levels of *E. coli* had increased over control values in the jejunum-ileum of all the stressed animals (Table 1). However, on the average, about one-half of the samples contained 10^7 or fewer bacteria per g. Before bacteria can be seen with ease in histological sections, about 10^7organisms per g of material must be present in the organ. Therefore, large numbers of organisms could have been seen only in the half of the sections from organs containing over that minimal population level.

In the sections from the stressed animals, gram-positive cocci and gram-negative rods dominated and were present between villi. By comparison, in sections from small bowels of control animals, bacteria were few in number and generally were seen to be restricted to the central luminal area.

A filamentous organism (C. P. Davis, S. L. Erlandsen, and D. C. Savage, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 57, 1973) was observed in sections of small bowel from control CD-1 and Ha/ICr mice. The incidence was somewhat reduced in mice infected with *Salmonella* but not deprived of food, water, or bedding. These filamentous microbes were rarely observed in stressed mice of either strain and were never observed in the C57BL animals (Table 6).

In Gram-stained histological sections of ceca and colons, the populations of fusiform-shaped bacteria normally associated with the mucosal epithelium of these organs were seen to be markedly reduced in stressed mice of all strains (Fig. 2c–f). In most cases, one-half to three-fourths of the animals in each stressed group showed this altered distribution. The fusiform-shaped bacteria had been replaced to some extent by gram-positive organisms.

In sections stained with hematoxylin and eosin, areas of edema could be seen at the junction of nonsecretory and secretory epithelia in the stomachs of the majority of stressed mice.

TABLE 4. *Summary of changes from normal in the aerobic gastrointestinal microflora of mice previously inoculated with S. typhimurium strain LT2 stressed with food, water, and bedding deprivation for 48 h*

Specimen	Consistent observation in stressed mice of all strains
Stomach	Decrease in lactobacilli
Jejunum + ileum	Increase in *Salmonella*
Cecum	Increase in *Salmonella* Increase in enterococci Increase in NLF[a] (CD-1)[b] Increase in *E. coli* (Ha/ICr) Increase in *Klebsiella* (C57BL)
Colon	Increase in *Salmonella* Increase in enterococci Increase in NLF (CD-1) Increase in *E. coli* (Ha/ICr) Increase in *Klebsiella* (C57BL)

[a] A non-lactose-fermenting variant of *E. coli*.
[b] Mouse strain in parentheses.

TABLE 5. *Anaerobic microflora of the large bowel of CD-1 mice stressed with food, water, and bedding deprivation*

Mice	Specimen	Group	Log_{10} viable count/g on	
			SEA[a]	BHIA[b]
CD-1	Cecum	Stress[c]	11 (9–11)[d]	11 (10–11)
		Control	11 (10–11)	11
	Colon	Stress	8 (8–9)	8 (8–9)
		Control	9 (9–10)	10 (9–11)
CD-1 (LT2)[e]	Cecum	Stress	10 (10–11)	11 (10–11)
		Control	11 (10–11)	11 (10–11)
	Colon	Stress	9 (8–9)	8 (8–10)
		Control	9 (8–11)	10 (9–11)

[a] Sweet E Agar.
[b] Brain heart infusion agar supplemented.
[c] Deprived of food, water, and bedding for 48 h.
[d] Median and range of population estimates; five mice per group.
[e] Mice previously inoculated with *S. typhimurium* strain LT2.

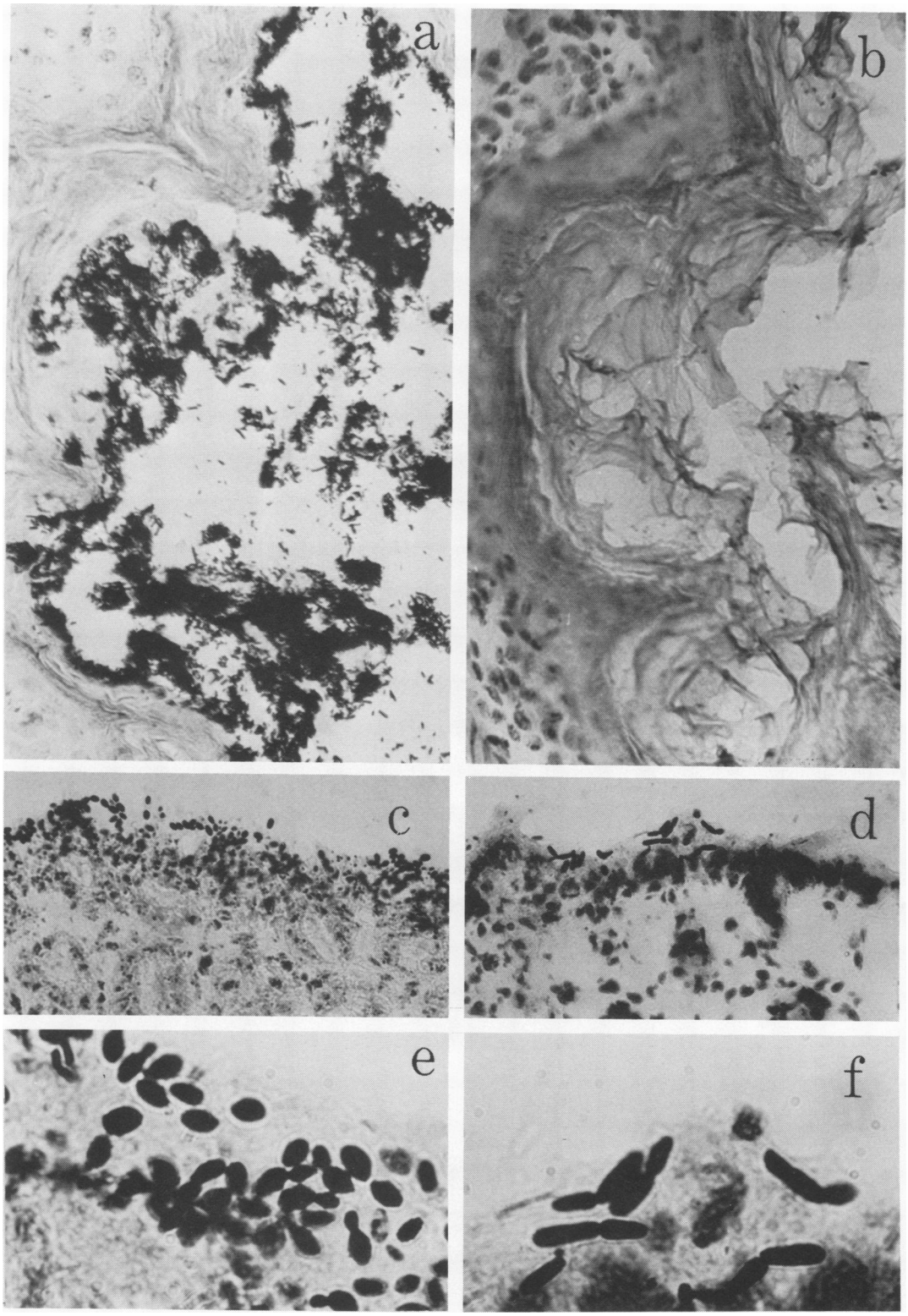

FIG. 1. *(a) Layers of gram-positive rods associated with the keratinized squamous epithelium of the stomach of a control CD-1 mouse; ×750. (b) Layers absent in stressed mouse stomach; ×750. (c) Yeast layers on secretory epithelium of stomach (C57BL control); ×750. (d) Scattered yeast cells in stressed mouse stomach; ×750. (e) Spherical to ellipsoidal yeast cells in C57BL control mouse stomach; ×3,000. (f) Elongated yeast cells in stressed mouse stomach; ×3,000. Gram stain.*

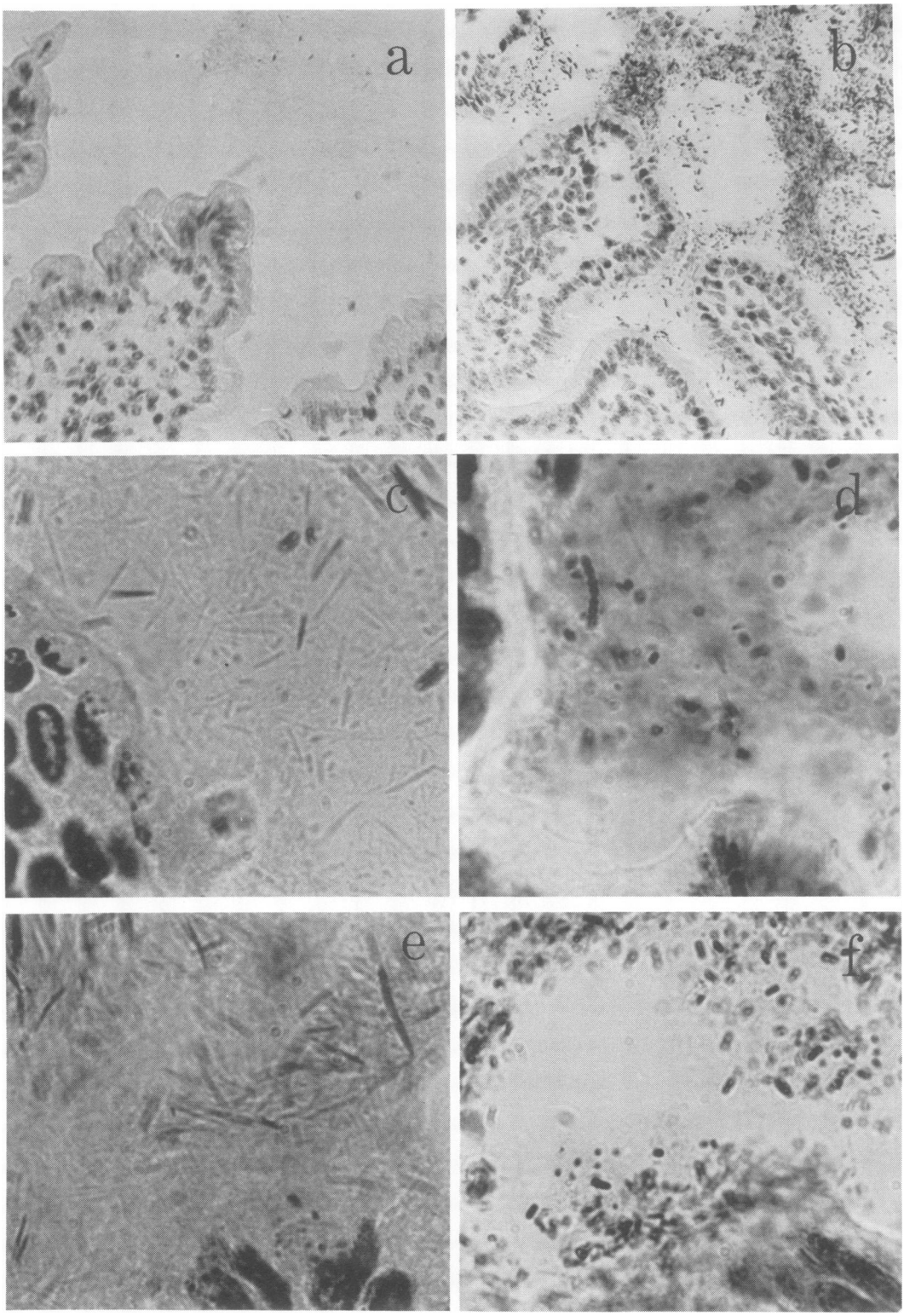

FIG. 2. *(a) Ileal section from control CD-1 mouse; ×750. (b) Microorganisms among villi of ileum in stressed mouse; ×750. (c) Fusiform-shaped bacteria predominate in region adjacent to cecal epithelium. Control Ha/ICr (LT2); ×3,000. (d) Fusiforms no longer predominate in stressed mouse cecum; ×3,000. (e) Fusiforms predominate on epithelium in colon of Ha/ICr control mouse; ×3,000. (f) Gram-positive organisms predominate near epithelium in stressed mouse colon; ×3,000. Gram stain.*

TABLE 6. *Incidence of mouse filamentous organism in histological sections of ileum*

Mouse strain	Group	Incidence[a]
CD-1	Stress	1/8
	Control	8/9
CD-1 (LT2)[b]	Stress	0/9
	Control	1/9
Ha/ICr	Stress	0/9
	Control	5/9
Ha/ICr (LT2)[b]	Stress	0/7
	Control	2/8
C57BL and C57BL (LT2)[b]	Stress	0/5
	Control	0/5

[a] Number of mice with filamentous organism in ileum/number of mice examined. One section examined per mouse.

[b] Mice previously inoculated with *S. typhimurium* strain LT2.

Enlarged Peyers patches could be seen in sections of the small and large bowels of both control and stressed mice previously infected with *Salmonella*.

DISCUSSION

Food, water, and bedding deprivation produced several marked changes in the indigenous microbiota of mice. The populations of lactobacilli normally colonizing the keratinized squamous epithelium of the stomach were dramatically reduced in the stressed animals. The yeast populations inhabiting the secretory epithelium of the stomach of C57BL control mice were also reduced in stressed animals. The yeasts of the genus *Torulopsis* (17) inhabiting the secretory epithelium have a spherical to ellipsoidal morphology. In stressed C57BL mice, however, the few yeast cells present were considerably elongated, reminiscent of rudimentary pseudohyphae. *Torulopsis* is described as producing elongated cells and a primitive pseudomycelium only rarely (13). The adverse conditions in the stomachs of stressed mice may induce the cells to form such structures. Alternatively, yeasts of more than one genus may be inhabiting the gastric epithelium.

In mice deprived of food, water, and bedding, the population levels of coliform bacteria increased in the jejunum-ileum and cecum of animals of all strains examined. In mice of two strains (CD-1, C57BL), coliform populations also increased in the colon. Such increases were accompanied by a rise in the numbers of enterococci. Coliforms and enterococci behave in a parallel manner during the establishment of the indigenous microbiota in baby mice (6, 20, 23).

From examination of histological sections of ceca and colons of stressed mice, we judged that the populations of fusiform-shaped bacteria normally inhabiting the mucosal epithelium in those areas of the bowel were markedly reduced. Interestingly, though, the estimates of the population levels of anaerobic bacteria in these intestinal areas were just as high in stressed as in control mice. Our counting technique, which cannot detect less than a 1-log difference between counts, may have been too insensitive to detect differences between the two groups. More likely, however, the habitat vacated by the fusiform-shaped microbes in the cecum and colon may have been filled by other bacterial types, thus maintaining the total anaerobic counts at control levels. The change in the relative distribution of cecal organisms is not unlike that described for mice given kanamycin orally for 24 h (19).

These changes in the indigenous microbiota obviously came about because the rigorous environmental and dietary stress imposed on the animals disrupted the mechanisms that regulate the localization and population levels of indigenous microbes in the stomach and bowel. For example, hormonal imbalance resulting from stress may lead to changes in the amount of mucous secreted into the gastrointestinal tract (4). It is likely that some indigenous microorganisms utilize carbohydrate moieties of gut mucins as a source of nutrition (8).

Impairment of the host immunological defenses can result as a consequence of hormonal imbalance and through inadequate diet or starvation (24). The influence of the host immunological system on the indigenous microbiota of the gastrointestinal tract is an unresolved question. The levels of natural serum antibodies do not appear to influence the population levels of *Escherichia coli* in the intestines of humans (14) and pigs (3). However, the effect of serum antibodies on other indigenous bacteria needs to be determined. The influence of secretory antibodies on indigenous microorganisms in the bowels is only in the early stages of investigation (5).

Other factors involved may include direct and indirect mechanisms of microbial interference. Direct interference may involve antibiotics produced by some bacterial strains, toxicity of metabolic products, and competition for specific attachment sites (18). Indirect interference involves host properties influenced by some members of the indigenous microbiota. For example, the microbiota stimulates antibody

production (25), the rate of migration of intestinal epithelial cells (9), and the motility of the small bowel (2). All of the factors may interact in complex ways to regulate microbial populations of the gastrointestinal tract.

Disruption of these regulatory mechanisms may well play a role in permitting *S. typhimurium* to multiply in the intestinal canal of mice in our experimental model. As previously described (27), the populations of *Salmonella* increase in the small and large bowels of mice deprived of food, water, and bedding for 2 days. These increased populations of *Salmonella* in the small bowel could have been due to an influx of organisms from systemic sites. But this seems unlikely at this early stage of infection. *Salmonella* population levels in organs other than the intestinal canal are still relatively low at 2 days after the stress situation in this study (27).

Thus, it is our opinion that the large *Salmonella* populations in the bowels of the stressed mice came about because factors involved in regulating the indigenous microbiota were disrupted by the complex stress imposed on the animals. The experimental model we have described is proving useful in investigating these factors.

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