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Localization of Translocating *Escherichia coli*, *Proteus mirabilis*, and *Enterococcus faecalis* within Cecal and Colonic Tissues of Monoassociated Mice

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Normal bacteria can translocate (migrate) across an intact intestinal mucosa, but the anatomical site of bacterial translocation has not been defined. Gastrointestinal (GI) cross-sections were obtained from mice monoassociated with high cecal concentrations of *Escherichia coli*, *Proteus mirabilis*, or *Enterococcus faecalis*. As previously reported (C. L. Wells, R. P. Jechorek, and K. J. Gillingham, Arch. Surg. 126:247–252, 1991), these mice had viable translocating bacteria recovered from mesenteric lymph nodes and livers. No abnormal GI histology was noted. Immunofluorescence was used to localize GI bacteria, and similar observations were made with each of the three bacterial species. Smaller numbers ($P < 0.01$) of bacteria were observed in stomach and small intestinal tissues than in cecal and colonic tissues, suggesting that the preferred site of tissue penetration for intestinal *E. coli*, *P. mirabilis*, and *E. faecalis* might be the cecum or colon as opposed to the stomach or small intestine.

Bacterial translocation has been proposed to cause many complicating infections in hospitalized immunosuppressed patients, trauma patients, and postsurgical patients (for a review, see reference 16). As defined by Alexander et al. (1), bacterial translocation is the passage of viable and nonviable microbes, as well as microbial components (such as endotoxin), across an anatomically intact intestinal barrier. In clinical studies, bacterial transport across an intact intestinal barrier has been difficult to confirm. However, studies involving animal models have supported the hypothesis that bacteria can translocate across intact intestinal tissue (1, 12–17). The primary purpose of the experiments reported herein was to clarify the gastrointestinal (GI) site(s) involved in bacterial translocation across histologically intact GI mucosae.

Although most studies have focused on the small intestine as the site of bacterial translocation (1, 6, 9, 10, 12, 14–17), other sites have not been systematically studied and cannot be ruled out. An ideal approach to elucidate the site of bacterial translocation would be to microscopically examine serial sections of entire GI tracts from animals with documented bacterial translocation. This approach is impractical. Herein, we have described immunofluorescent localization of bacteria within defined GI segments excised from germfree mice that had been monoassociated with *Escherichia coli*, *Proteus mirabilis*, or *Enterococcus faecalis*. These intestinal species do not typically translocate in animals with normal intestinal floras (1, 6, 8, 12, 14–16). A reliable way to study translocation of these species is to induce intestinal overgrowth (16). The monoassociated mice not only had reliably high numbers of intestinal bacteria but also permitted immunofluorescent localization of known bacterial species in the absence of the cross-reactive antigens present in a normal intestinal microflora.

Male and female, 6- to 7-week-old, germfree BALB/c mice were bred and housed at the University of Wisconsin Gnotobiotic Laboratory, Madison. Equal numbers of T-cell-deficient athymic mice and their functionally normal littermates were used in these studies. Male and female mice were housed in separate cages, and only littermates were housed together. *P. mirabilis* M13, *E. coli* M14, and *E. faecalis* M20 had been isolated from mouse mesenteric lymph nodes during previous studies of bacterial translocation in our laboratory (14, 15). An overnight tryptic soy broth (Difco Laboratories, Detroit, Mich.) culture of each species was placed in a separate germfree isolator, and mice were monoassociated by placing the organism in the drinking water. As previously reported (13), each bacterial species colonized the ceca at abnormally high concentrations (10^{10} to 10^{11}/g), no extraneous contaminating bacteria were detected in the cecal floras, and viable translocating bacteria were recovered from mesenteric lymph nodes and livers. The present morphological study involved 24 monoassociated mice, i.e., 8 mice monoassociated with *P. mirabilis*, *E. coli*, or *E. faecalis*. There were equal numbers of male and female mice and equal numbers of congenitally athymic mice and their immunocompetent littermates.

Seven days after monoassociation, mice were killed by CO_2 asphyxiation and eight GI segments were excised from each of the 24 mice, for a total of 192 tissue segments representing the following: stomach (keratinized and secreting portions), duodenum (1 cm distal to the stomach), jejunum (1 cm distal to the ligament of Treitz), ileum (1 cm proximal to the ileocecal valve), cecum (a central portion), proximal colon (1 cm distal to the cecum), and distal colon (1 cm proximal to the anus). Adjacent tissue samples from each site were placed in buffered formalin (for hematoxylin and eosin staining) and in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (for immunofluorescent localization of bacteria).

New Zealand White rabbits (Birchwood Valley Farms, Redwing, Minn.) were used to raise antisera to *E. coli* M14, *P. mirabilis* M13, or *E. faecalis* M20. Preimmune sera

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contained no demonstrable antibodies to these bacteria as measured by indirect immunofluorescence with affinity-purified, fluorescein-conjugated goat anti-rabbit immunoglobulin G (Organonteknika-Cappel, West Chester, Pa.) as the detector. Rabbits were injected intravenously, three times per week for 5 weeks, with 5×10^9 heat-killed (60°C, 1 h) bacteria suspended in 1 ml of sterile saline. Antisera were diluted in phosphate-buffered saline containing 5% goat serum and 0.05% sodium azide. *E. coli*, *P. mirabilis*, and *E. faecalis* antisera were used at dilutions of 1:3,000, 1:6,000, and 1:1,000, respectively.

The pattern of antiserum reactivity with bacterial surface structures (cell wall, fimbriae, flagella) was observed by immunogold labelling. Overnight tryptic soy broth cultures of *E. coli*, *P. mirabilis*, and *E. faecalis* were washed, placed for 30 min at room temperature on separate glass chips precoated with 0.1% poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.), rinsed with saline, reacted with the appropriate rabbit antiserum for 30 min at room temperature, rinsed with saline, incubated with affinity-purified goat antiserum to rabbit immunoglobulin conjugated to 10-nm gold particles (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) for 30 min at room temperature, rinsed with saline, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in ethanol, and critical-point dried by the CO_2 method of Anderson (2). A saddle field ion beam gun (Ion Tech, Ltd., Middlesex, England) was used to coat the bacteria with a 1-nm discontinuous layer of platinum. These preparations were examined at 4 kV with a Hitachi S-900 field emission scanning electron microscope by using a backscatter detector to enhance the visualization of colloidal gold by atomic number contrast. Immunogold labelling revealed that the antisera reacted with the cell wall of *E. coli*, *P. mirabilis*, or *E. faecalis* and with the flagella and fimbriae associated with the two gram-negative species (Fig. 1a to c).

In initial studies, paraffin-embedded tissues (preserved in formalin) were processed for immunofluorescent localization of bacteria as previously described (12). After observing the GI sections in this manner, we could not completely eliminate the possibility of artifactual movement of bacteria during tissue sectioning. Adjacent GI tissues (preserved in glutaraldehyde) were subsequently embedded in JB-4 plastic resin (Polysciences Inc., Warrington, Pa.), cut into 1.5-μm cross-sections, reacted with the appropriate rabbit antiserum for 16 to 18 h at 4°C, incubated with the fluorescein-conjugated anti-rabbit immunoglobulin G antibody for 20 min at room temperature, mounted in phosphate-buffered saline–glycerol (1:9) containing 0.1% phenylenediamine (Sigma) at pH 8, and viewed by epifluorescence microscopy. Entire tissue cross-sections were examined, as opposed to representative areas. GI tissue sections were also labelled with immunogold, and scanning electron microscopic observations indicated that the bacterial cells were firmly polymerized within the plastic resin and that the antisera reacted exclusively with cell wall material (Fig. 1d). Thus, if cut in the optimal plane of sectioning, immunofluorescent bacteria should appear as uniform ovals or circles within GI tissue.

No gross pathology was evident at autopsy, and hematoxylin and eosin staining of GI tissue revealed no abnormal histology. Immunofluorescence studies of GI tissue revealed similar results from male and female and from athymic and immunocompetent mice. (This latter observation was expected because Maddaus et al. [8] reported similar incidences of bacterial translocation in athymic and immunocompetent mice.) The immunofluorescence results have therefore been presented according to the species of bacteria.

Luminal and adherent bacteria were enumerated and scored semiquantitatively on the basis of the average numbers of bacteria observed per high-power field (40× objective): 1+, 1 to 10 bacteria; 2+, 11 to 100 bacteria; 3+, 100 to 400 bacteria; 4+, >400 bacteria. As expected, luminal bacteria were relatively sparse in the upper regions of the GI tract but became increasingly numerous in the lower GI tract. Luminal bacteria ranged from 0 to 2+ in the stomach and duodenum, from 0 to 3+ in the jejunum and ileum, and from 0 to 4+ in the cecum and colon. Luminal bacteria appeared to preferentially colonize intestinal debri and mucus (Fig. 2a). In general, adherent bacteria were also relatively sparse in the upper regions of the GI tract. In the small intestine, the majority of adherent bacteria were associated with the villus, and only rarely were bacteria adherent to the crypt epithelium. Adherent bacteria became increasingly dense in the lower portions of the GI tract, especially the cecum and colon, in which it was not unusual to observe dense bacterial masses adherent to the epithelium (Fig. 2b). (Photographs such as Fig. 2b also supported the concept that bacteria embedded in plastic resin were not artifactually carried across the intestinal tissue during tissue sectioning.) Masses of adherent bacteria were never observed in the small intestine, even in the region of the terminal ileum. There were no obvious differences in the numbers (or patterns) of luminal and adherent bacteria in the GI tracts of mice monoassociated with *E. coli*, *P. mirabilis*, or *E. faecalis*. Although much animal-to-animal variation was noted, the GI segments were not subjected to in situ fixation, and this variability might have been artifactual.

Bacteria within intestinal tissue were enumerated as the total number per millimeter; outer diameters of mounted tissue cross-sections were measured with a vernier caliper, and the circumferences were calculated to determine the number of millimeters of mucosa examined (Table 1). In all groups of monoassociated mice, although five segments of upper intestine were examined as opposed to three segments of lower intestine, the numbers of bacteria were larger in the lower GI tract (cecum and colon) than in the upper GI tract (stomach and small intestine). In all groups, fewer bacteria were observed within the epithelium than in the deeper regions of the mucosa, i.e., lamina propria, submucosa, and muscularis externa. In each group of monoassociated mice, the numbers of bacteria in the lower intestine were larger than those in the upper intestine (Table 1). Additionally, the numbers of bacteria in the lower GI tracts of *E. faecalis*-monoassociated mice were larger than those in the lower GI tracts of *E. coli*- or *P. mirabilis*-monoassociated mice (Table 1). In a previous study (which included the same mice used for the present study), the incidence of systemic infection (viable bacteria recovered from the liver) was significantly greater among *E. faecalis*-monoassociated mice than among *E. coli*- or *P. mirabilis*-monoassociated mice (13). Perhaps the increased number of bacteria observed within the cecal and colonic tissues of *E. faecalis*-monoassociated mice was related to the relative invasive potential of *E. faecalis*.

Figure 2c and d demonstrate the appearance of bacteria within cecal tissues of monoassociated mice. Although some immunofluorescent bacteria appeared to be within vessels, the tissue segments were unstained and vessel localization could not be confirmed with certainty. We have previously proposed that intestinal bacteria may translocate within tissue phagocytes (16), but that hypothesis could be neither supported nor refuted by these observations. Although rel-

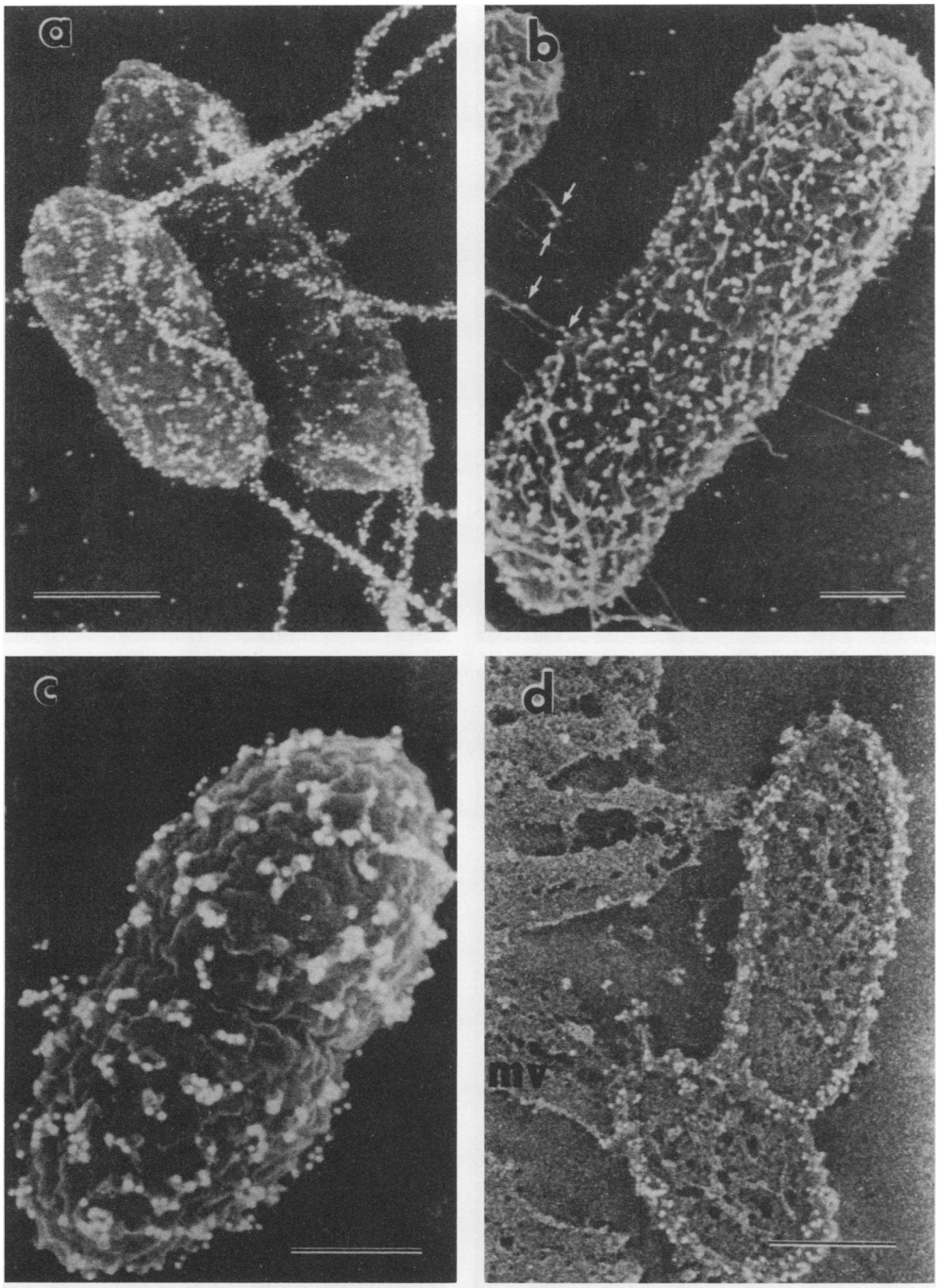

FIG. 1. Immunogold labelling used to visualize antiserum reactivity with the cell surfaces of *P. mirabilis*, *E. coli*, and *E. faecalis*. (a) Two *P. mirabilis* cells with gold particles on the cell wall and flagella; (b) an *E. coli* cell with gold particles on the cell wall and fimbriae (arrows); (c) a diplococcal form of *E. faecalis* with gold particles on the cell wall; (d) colonic microvilli (mv) from a mouse monoassociated with *P. mirabilis*; individual bacterial cells appear in cross section, with the gold labelling concentrated on the cell wall material. Bars in panels a and d = 0.5 μm, and those in panels b and c = 0.25 μm.

atively few bacteria were observed to be associated with epithelial cells, these bacteria appeared to be within rather than between epithelial cells, suggesting transcellular rather than paracellular transport of translocating bacteria.

In discussing these results obtained by using monoassociated mice, the following caveats concerning the use of germfree animals should be mentioned. It has been suggested that the germfree animal may not be a relevant animal model because of its relatively unprimed immune system. Compared with conventionally raised animals, germfree animals have little detectable gamma globulin (3) and less lymphoid tissue (7). However, after monoassociation or after stimulation with specific antigen(s), germfree animals exhibit lymphoid tissue proliferation and immune responses

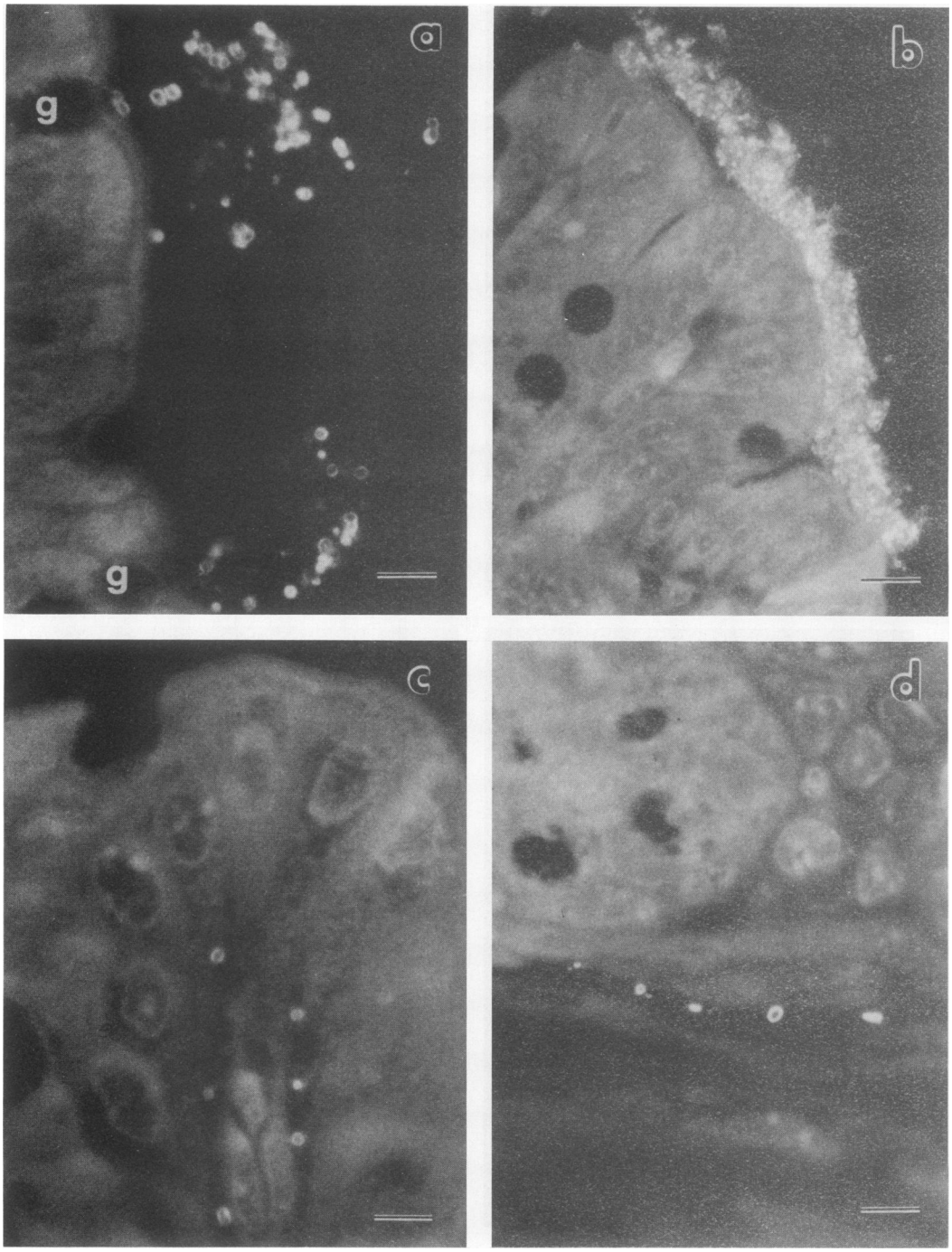

FIG. 2. Immunofluorescent localization of bacteria in cecal and colonic tissues from monoassociated mice. (a) Luminal *E. faecalis* appearing to preferentially stick to mucus that is being discharged from two colonic goblet cells (g); (b) dense mass of *E. coli* adherent to the colonic epithelium; (c) *E. faecalis* below the epithelium and within the cecal lamina propria; (d) *P. mirabilis* within the cecal submucosa. Bars in panels a, c, and d = 5 μm, and that in panel b = 10 μm.

that are similar in degree to those of their conventionally raised counterparts (3–5, 7, 11). Hematoxylin and eosin staining of all GI segments examined in this study revealed normal cellular architecture, including normal lymphoid follicles and prominent Peyer's patches.

The results of this study suggested that, in monoassociated mice with histologically normal intestinal mucosas, the preferred site for translocation of *E. coli*, *P. mirabilis*, and *E. faecalis* might be the cecum or colon as opposed to the stomach or small intestine. Although additional studies with other animal models are needed to confirm these findings, these results indicated that the large intestine should not be excluded from future histological studies of bacterial translocation. Information concerning the anatomical route of

TABLE 1. Total numbers of immunofluorescent bacteria in the upper and lower GI tracts of eight mice monoassociated with *E. coli*, *P. mirabilis*, or *E. faecalis*[a]

Monoassociating organism	Total no. of bacteria observed in tissue cross-sections from[b]:					
	Upper GI tract			Lower GI tract		
	No. of bacteria	Tissue diameter (mm)	Ratio	No. of bacteria	Tissue diameter (mm)	Ratio
E. coli	2	311	0.01	61	219	0.28[c]
P. mirabilis	10	306	0.03	41	230	0.18[c]
E. faecalis	6	308	0.02	102	192	0.53[c,d]
Total	18	925	0.02	204	641	0.32[c]

[a] Upper GI tract includes keratinized stomach, secreting stomach, duodenum, jejunum, and ileum; lower GI tract includes cecum, proximal colon, and distal colon.

[b] Ratios are the numbers of bacteria per millimeter of tissue.

[c] Increased compared with the number of bacteria in the upper GI tracts of mice monoassociated with the corresponding species of bacteria; $P < 0.01$ by chi square.

[d] Increased compared with the numbers of bacteria in the lower GI tracts of *E. coli*- or *P. mirabilis*-monoassociated mice; $P < 0.01$ by chi square.

bacterial translocation should be useful in designing more mechanistic studies of this process.

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