Effects of Commonly Used Bowel Preparations on the Large Bowel Mucosal-Associated and Luminal Microflora in the Rat Model

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Studies of colonic microflora have indicated there are two distinct populations, one intraluminal and one mucosal surface-associated. This investigation further characterizes the mucosal surface microflora and assesses the effects of common preoperative bowel preparations on both microflora. Quantitative and qualitative bacterial cultures and scanning electron microscopy were used to study the microflora in five groups of seven rats each: control; intraoperative colonic instillation of ten percent povidone-iodine for 20 minutes; mechanical preparation with magnesium citrate; mechanical preparation followed by intramuscular cefoxitin (30 milligrams per kilogram) one hour preoperatively; and mechanical preparation followed by oral neomycin sulfate and erythromycin base (15 milligrams/kilogram each) given by gavage tube 18, 14, and 4 hours preoperatively. Microflora on the mucosal surface was visualized by scanning electron microscopy in all groups except the neomycin/erythromycin group. Results showed fewer bacterial isolates recovered from the mucosal surface compared with the lumen, as well as several $\log_{10}$ units lower for each bacterial classification. The greatest suppression of both microflora was seen in the neomycin/erythromycin group. Total aerobic and anaerobic luminal counts decreased by 3.7 ($P < 0.009$) and 6.3 ($P < 0.009$) $\log_{10}$ units, while total aerobic and anaerobic wall counts decreased by 2.3 ($P < 0.009$) and 2.8 (not significant) $\log_{10}$ units, respectively. Lesser reductions were noted in the povidone-iodine group ($P < 0.009$, $P < 0.009$, $P < 0.009$, and $P < 0.048$, respectively). There were no statistically significant reductions in either total aerobic or anaerobic counts in the mechanical preparation or cefoxitin groups. These results indicate that neomycin/erythromycin is the most effective regimen in reducing both microflora.

[Key words: Colonic flora; Bowel preparation; Mucosal-associated and luminal microflora]

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THE ASSOCIATION OF microorganisms with the gastrointestinal mucosa has been studied with increasing interest in recent years.[1-5] Lee and others[6] have defined two types of ecologic niches occupied by microorganisms in the mammalian gastrointestinal tract: a mucosal-associated autochthonous flora and a second found within the luminal content. A mucosal-associated organism has previously been defined as any organism that can be identified in significant numbers in

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specimens of intestinal tissue after vigorous washing.[6-7] Autochthonous microbes are characterized as indigenous microorganisms that colonize particular regions of the gastrointestinal tract early in life, multiply to high population levels soon after colonization, and remain at those levels in a symbiotic relationship with the host.[7-8] Scanning electron microscopy has been used to visualize these microbes populating the epithelial surfaces in the gastrointestinal tracts of mice and humans.[2,9] The degree of similarity of the mucosal-associated and luminal microflora of the colon has not been extensively studied and is one of the interests of this investigation.

The classic example of bacterial adherence is the attachment of the fimbriae or common pili of gram-negative rods to the epithelial surface.[10] These long slender structures originate from the bacterial cell membrane, extend through the cell wall, and project from the cell surface. For organisms that lack these physical structures and for the organisms found within the extensive film of mucous that lines the bowel, a second mechanism of adhesion has been described. This adhesion has been shown to be due to a temporary molecular or Stefan adhesion of the bacteria to surfaces.[6,11] Fluid dynamics used to quantitate these adhesive forces for Flexibacter BH3 have shown that the force preventing separation from a surface is very much greater than the horizontal drag through a viscous medium.[11-12] In addition to their known adhesive properties, these organisms associate with the mucosal surface in other ways such as survival and multiplication within the mucous layer lining the epithelial surface, and metabolism of mucins for energy.[6]

To date, studies of large bowel antisepsis have generally relied on intraoperative sampling of the luminal contents alone.[13] The effects observed on the luminal microflora have traditionally been assumed to also occur on the mucosal-associated microflora. This theory has recently been challenged by Rotstein *et al.*,[14] who found that the mucosal-associated bacteria may be relatively protected from the bactericidal activity of povidone-iodine, and thus prompted this investigation of the effectiveness of various bowel preparations against both the mucosal-associated and luminal microflora.

Materials and Methods

Experimental Animals: Male Sprague-Dawley rats weighing from 650 to 750 gm were obtained from Charles-River, Inc. (Wilmington, Massachusetts). The Public Health Service "Guide for the Care and Use of Laboratory Animals" and the guidelines of the Animal Welfare Act were followed. Approval for this study was obtained from the Advisory Committee for Animal Resources at Tulane University.

Treatments: The treatment groups consisted of five groups of seven rats each: untreated control, intraoperative colonic instillation of ten percent povidone-iodine for 20 minutes (PI); mechanical preparation with magnesium citrate (MECH); mechanical preparation followed by intramuscular cefoxitin (30 milligrams/kilogram) one hour preoperatively (FOX); and mechanical preparation followed by oral neomycin sulfate and erythromycin base (15 milligrams/kilogram each) given by gavage tube at 18, 14, and 4 hours preoperatively (NE). The dosage of cefoxitin was calculated as 30 milligrams/kilogram to be approximately proportional to a 2-gm dose in an average adult human. The rat was chosen for this protocol because it has been shown that absorption, bioavailability, and metabolism of cefoxitin are the same as in humans.[15] Virtually the entire dose of cefoxitin is available to the systemic circulation after intramuscular administration with peak serum levels being achieved in 20 to 30 minutes. In the NE group, the dose of oral neomycin sulfate was 15 milligrams/kilogram, approximately proportional to a 1-gm dose in an average adult human. The dose of erythromycin base was computed similarly. Neomycin sulfate and erythromycin base are poorly to minimally absorbed through the gastrointestinal tract, and the effects of these agents on the colonic microflora are mainly due to their direct local effects. In the PI group, the 20-minute exposure to ten percent povidone-iodine was representative of an average amount of time necessary to mobilize a segment of the colon in an emergency operation.[16-17] In the FOX, NE, and MECH groups, the rats were restricted to water ad libitum for 24 hours followed by catharsis.

The catharsis (5.8 percent solution of magnesium citrate in conjunction with a 70 percent sorbitol solution) was administered by feeding bottle until the stool was watery. The preparation period and the total dosage varied for each rat, ranging from 36 to 48 hours and 67 to 94 ml, respectively. All rats were fed a 50 percent beef diet for at least 15 days because this diet increases the fecal aerobes and anaerobes.[18]

Operative Procedure and Bacteriology: All animals were sacrificed with carbon dioxide gas and then underwent immediate laparotomy. Under sterile operating conditions, the ceca were milked free of contents, ligated at the ileocecal junction and proximal ascending colon, and injected with 3 ml of sterile saline in all groups except the PI group, where 3 ml of 10 percent povidone-iodine was used. After a 20-minute dwell time, the luminal contents and cecal wall were each cultured for viable bacteria by the research bacteriology laboratory. A needle and syringe was used to aspirate 1.1 ml of luminal contents, and a template was used to harvest sections of cecal wall 1.54 cm^2 (1.40

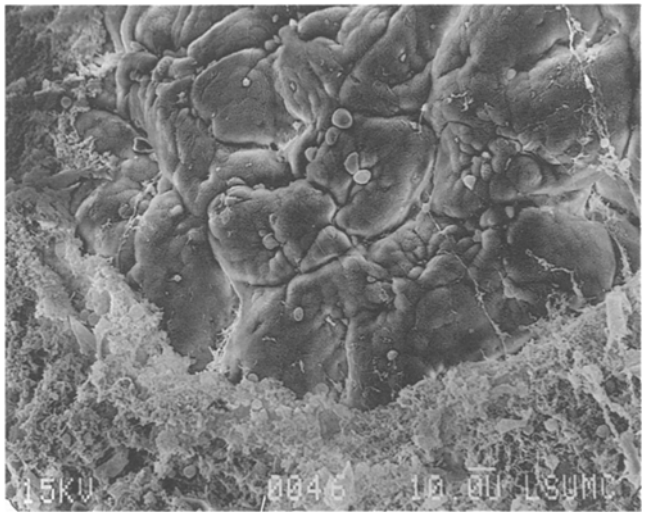

FIG. 1. Low power scanning electron micrograph of rat large bowel mucosa (FOX ×440). The mucous blanket appears approximately 10 to 15 micrometers thick at the interface with the epithelial surface. Mucous plugs can be seen within the crypts of Lieberkühn. This blanket structure was present in all study groups.

centimeter diameter circle). The tissue specimens were irrigated with sterile saline to remove all traces of residual fecal material and then homogenized in 3 ml of sterile saline utilizing an Omni-Mixer®. The homogenized tissue and the luminal contents were serially diluted with prereduced dilution fluid in an anaerobic glove box chamber (Coy Laboratories, Ann Arbor, Michigan). The small amount of residual iodine was not neutralized due to the extent of the dilutions carried out and the findings of the same numbers of bacteria as in control during preliminary studies. Anaerobic cultures were performed by plating on vitamin K-hemin and kanamycin-vancomycin blood agars. Anaerobic cultures were performed by using tryptic soy agar with five percent sheep blood, eosin methylene blue, bile esculin azide, and mannitol salt agars. All cultures were incubated 24 to 48 hours at 35° C. (aerobic and anaerobic). Aerobic gram-negative bacteria were identified biochemically using the Enterotube® and Oxi/Ferm® systems and aerobic gram-positives with standard biochemical tests. Anaerobic organisms were identified with a Rapid Ana Test Kit®.

Scanning Electron Microscopy: The cecal tissue from two rats in each treatment group was prepared for scanning electron microscopy. All tissue specimens were irrigated as mentioned above, prefixed in 2 percent gluteraldehyde for two to four hours, immersed in cacodylate buffer (pH 7.2) for two to four hours, and then serially dehydrated in acetone. The specimens were dried in a Samdri-790 Critical Point Dryer (Tousimis Research Corp., Rockville, Maryland) using liquid carbon dioxide and then gold coated by a Hummer VI sputtering system coater (Anatech, Ltd., Alexandria, Virginia). The specimens were viewed with a JEOL JSM-35CF scanning electron microscope (JEOL Corp., Teabody, Massachusetts).

Statistical Methods: Geometric means were used to compare the bacterial classifications in each treatment group. Because of the nonparametric distribution of the data, further comparisons between the treatment groups were made utilizing the Kruskal-Wallis test.

Results

At the outset of this investigation, the samples of cecal tissue were overwashed with resultant de-epithelization of the mucosal surface and extremely low bacterial counts. After alteration of the experimental protocol, the washing technique that resulted in the least artifact and the most consistent qualitative and quantitative bacteriologic results was gentle irrigation of each tissue sample with sterile normal saline (one to two ml) until only the shiny mucosal surface was grossly visible.

The scanning electron microscopy demonstrated a mucous blanket adherent to the epithelial surface for all groups (Fig. 1), varying in thickness from 5 to 15 fm. The different experimental treatments did not appear to remove or otherwise alter the structure of this blanket. A dense population of bacteria was clearly visible within the mucous blanket or directly attached to the mucosal surface in all study groups except the NE group (Fig. 2A-D). These findings were consistent in the 30 specimens scanned among the five different treatment groups.

Because of the diversity of bacterial species isolated from the different treatment groups, bacteria were grouped into related classifications to enable further between-group comparisons. In the control group, as in all treatment groups, a greater variety of bacterial isolates were recovered from the large bowel lumen when compared with the mucosal surface. All isolates were typical colonic flora, and the predominant isolates in control (consistently isolated in counts greater than 10^4 colony-forming units per cm^2 mucosa or greater than 10^6 colony-forming units per ml luminal fluid) are listed in Table 1. Not all of the predominant isolates in control appeared to be mucosal-associated, as *Proteus* sp, *Clostridium* sp, and several species of *Bacteroides* were below the lower limits of detectability on the mucosal surface (less than 3×10^3 colony-forming units per cm^2 mucosa).

A quantitative comparison of the mucosal and luminal microflora in controls was also undertaken using the same related classifications. When the mucosal counts (colony-forming units per cm^2 muscosa) were plotted against the luminal counts (colony-forming units per ml luminal fluid), the mucosal counts were generally

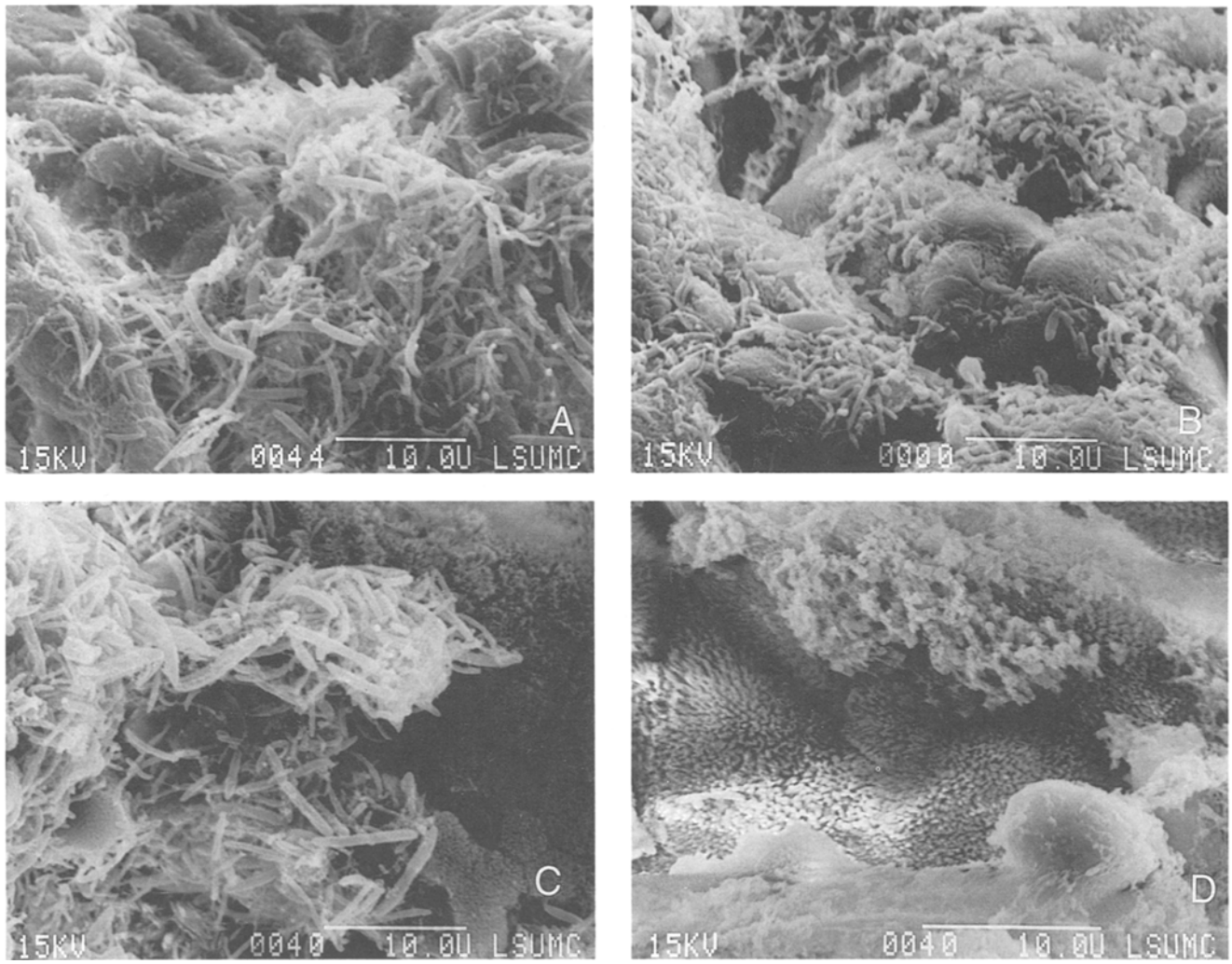

FIG. 2. Mucosal-associated microorganisms can be seen in all study groups except NE. A. (Control ×2200) A plethora of rod-shaped organisms are exuding from the crypt of Lieberkühn and extending out onto the large bowel surface. The intercellular clefts are well demonstrated in this electron-micrograph. A portion of basement membrane, visible in the lower left-hand portion of this micrograph, has been inadvertently denuded of epithelial cells, most likely due to the washing process. B. (FOX ×2200) The mucous blanket is tenuous enough in this area to allow visualization of the bacteria that appear to be directly attached to the mucosal surface, while other bacteria appear to be more loosely associated with the surface in the overlying mucous blanket. C. (Control ×2400) The bacteria are again demonstrated within the mucous blanket at this interface with the epithelial surface. The hexagonal borders of the individual epithelial cells can be seen on the right-hand portion of this micrograph at this magnification. D. (NE ×3000) Microorganisms cannot be identified either on the velvety brush border of the large bowel mucosal surface nor within the overlying mucous blanket.

found to be several $\log_{10}$ units lower than the corresponding luminal counts (Fig. 3).

The greatest suppression across all classes of bacteria (Table 2), both on the mucosal surface and from the cecal lumen, occurred in the NE group when compared with controls (Fig. 4A-B).

The luminal suppression in the NE group ranged from a low of 3.0 $\log_{10}$ units for aerobic gram-negative rods ($P < 0.009$) to a high of 8.4 $\log_{10}$ units for *Bacteroides* sp ($P < 0.009$). The mucosal-associated bacterial suppression ranged from a low of 2.1 $\log_{10}$ units for anaerobic organisms other than *Bacteroides* sp (not significant) to a high of 4.6 $\log_{10}$ units for aerobic gram-positive cocci ($P < 0.009$).

Povidone-iodine treated animals also showed statistically significant reductions in the luminal- and mucosal-associated counts for a majority of the bacterial classifications. These reductions, however, were not as marked as those seen in the NE group. The luminal bacterial suppression in the PI group ranged from a low of 1.1 $\log_{10}$ units for aerobic gram-negative rods (not significant) to a high of 3.7 $\log_{10}$ units for *Bacteroides* sp ($P < 0.009$). The mucosal-associated bacterial suppression ranged from a low of 1.9 $\log_{10}$ units for anaerobes other than *Bacteroides* sp (not significant) to a high of 2.8 $\log_{10}$ units for aerobic gram-negative rods ($P < 0.048$).

Magnesium citrate had little effect on the microflora, achieving statistically significant reductions only in the

TABLE 1. *Predominant Isolates for Each Classification of Bacteria in Control Group*

Bacterial Classification	Luminal Isolates	Mucosal Isolates
Aerobic gram-negative rods	*Escherichia coli*	*E. coli*
	Klebsiella sp	*Klebsiella* sp
	Morganella sp	*Morganella* sp
	Proteus sp	
Aerobic gram-positive cocci	Enterococcus	Enterococcus
	Coagulase-positive	Coagulase-positive
	Staphylococcus	Staphylococcus
	Coagulase-negative	Coagulase-negative
	Staphylococcus	Staphylococcus
Aerobic gram-positive rods	*Bacillus* sp	*Bacillus* sp
	Lactobacillus sp	*Lactobacillus* sp
Bacteroides sp	*Bacteroides fragilis*	*B. fragilis*
	Bacteroides thetaiotaomicron	*B. thetaiotaomicron*
	Bacteroides melaninogenicus	
	Bacteroides asaccharolyticus	
	Bacteroides distasonis	
	Bacteroides ovatus	
Anaerobes other than *Bacteroides* sp	*Fusobacterium varium*	*F. varium*
	Eubacterium aerofaciens	*E. aerofaciens*
	Acinetobacter odontolyticus	*A. odontolyticus*
	Clostidium perfringens	
	Clostidium subterminale	
	Clostidium clostridiiforme	

mucosal-associated and luminal aerobic gram-positive rods ($P < 0.048$ and $P < 0.009$, respectively). Cefoxitin was shown to be even less effective in reducing the microflora, achieving a statistically significant reduction in only the luminal aerobic gram-positive rods ($P < 0.048$).

Discussion

The intestinal mucous blanket has long been recognized as a first-line immunologic defense mechanism for the host. Mucin granules have the ability to absorb viruses, and secretory IgA and other immunoglobulins adhere to epithelial cell surfaces, project into the overlying mucous, and provide an "antiseptic paint" that can block adherence of pathogenic agents to epithelial cells and neutralize their toxins.[19] The autochthonous flora of the bowel is known to be antagonistic to enteric pathogens and includes both the luminal microflora as well as the flora that can be readily visualized within the mucous blanket and directly adherent to the mucosal surface. Conversely, this mucosal-associated microflora may also be a reservoir for endogenous organisms during colon surgery and may adversely effect anastamotic healing. The role that the mucosal microflora may play in the recently reported translocation of bacteria across the gut mucosal barrier

TABLE 2. *Reductions in Bacterial Counts for Each Treatment Group (log_{10} units) Compared with Control*

	NE		PI		MECH		FOX	
Bacterial Classification	Lumen	Wall	Lumen	Wall	Lumen	Wall	Lumen	Wall
Total Aerobic	3.7*	2.3*	1.5*	2.0*	NS‡	NS	NS	NS
Gram-negative rods	3.0*	3.5*	NS	2.8†	NS	(2.6)§	NS	NS
Gram-positive cocci	4.3*	4.6*	NS	NS	NS	(1.0)§	NS	NS
Gram-positive rods	4.3*	3.0*	3.3*	2.1*	0.4*	2.3†	1.4†	NS
Total Anaerobic	6.3*	NS	3.2*	2.9†	NS	NS	NS	NS
Bacteroides sp	8.4*	NS	3.7*	NS	NS	NS	NS	NS
Other anaerobes	NS	NS	NS	NS	NS	NS	NS	NS

*$P < 0.009$.
†$P < 0.048$.
‡NS: not significant ($P > 0.05$).
§Counts were significantly increased compared with CON ($P < 0.009$).
Differences of the geometric means of the groups and compared by the Kruskal-Wallis test.

in stressed or traumatized animals has yet to be defined.[20]

Postoperative infections after colon surgery are generally due to contamination from the endogenous flora.[21-22] Clinical studies have proven that neomycin sulfate and erythromycin base effectively suppress the luminal microflora.[23] This investigation shows that in our animal model, the autochthonous mucosal-associated bacteria are also suppressed. Scanning electron microscopy confirmed the bacteriologic results, showing few if any organisms within the mucous blanket or attached to the mucosal surface in the NE treated animals.

This study corroborates the previous descriptions of the mucosal-associated microflora.[24-29] We do, however, extend our observations to note that the mechanical preparation alone neither changed the appearance of the mucosal-associated bacteria, as shown by scanning electron microscopy, nor appreciably altered the luminal- or mucosal-associated bacterial counts except for small but statistically significant decreases in aerobic gram-positive rods. These data seem plausible as previous investigations into the effects of catharsis on the luminal microflora have shown no marked changes in the bacterial flora counts.[21,30]

There has been no study of the immediate (less than 60 minutes) effect of a single preoperative dose of a cephalosporin on the intestinal microflora.[31] We report from this investigation little if any suppression except for a small but statistically significant reduction in luminal aerobic gram-positive rods. This reduction, however, is more likely attributable to the catharsis and not the antibiotic, as similar effects occurred in animals treated with the mechanical preparation alone. The spectrum of activity of cefoxitin includes many of the common enteric organisms; thus, the antibiotic must fail to penetrate to the mucosal and luminal microflora or may be inactivated by the mucus.

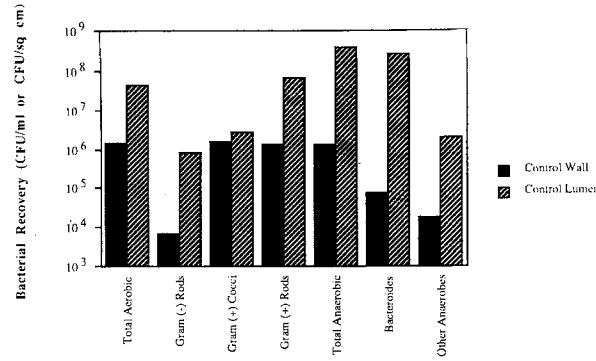

FIG. 3. Luminal *vs.* mucosal bacterial counts in control group.

Although povidone-iodine achieved significant reductions in the intestinal microflora, these reductions were less than expected. Microorganisms could still be seen densely adherent to the mucosal surface, and bacterial recovery from the mucosal surface was substantial. The only explanation that we can offer for this finding is that the 20-minute exposure to 10 percent povidone-iodine was not sufficient for killing many bacteria, for lysis of the bacterial cell walls, or for disruption of attachment mechanisms to occur.

Summary

In this experimental study, preoperative mechanical preparation followed by oral neomycin sulfate and erythromycin base was the most effective regimen in reducing both the mucosal-associated and luminal microflora. This dual suppression may in part explain the widespread successful clinical experiences noted with this bowel preparation over the last 15 years. For this reason, we continue to advocate this regiment before elective colorectal surgery. Intraoperative instillation of 10 percent povidone-iodine into the colonic lumen may be useful for emergent surgery.

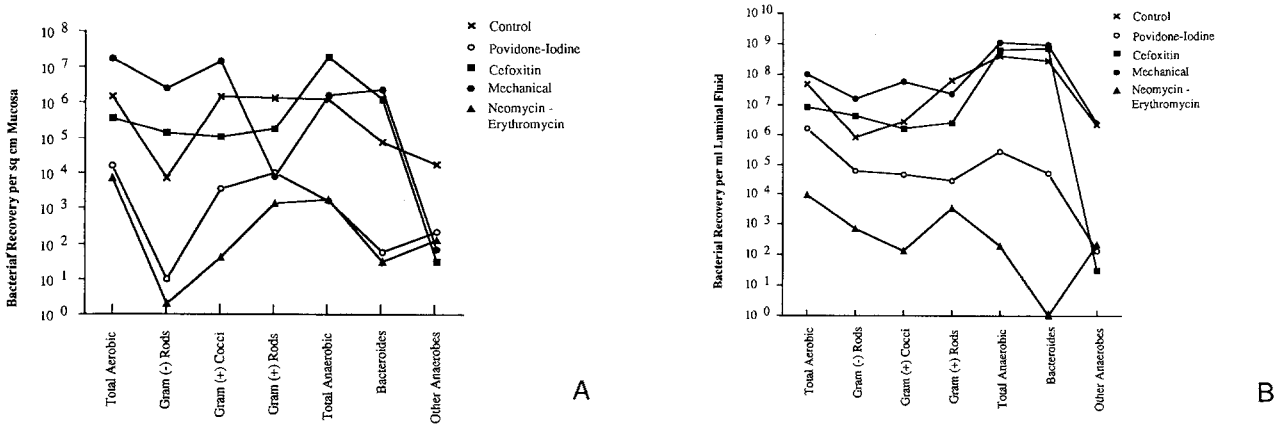

FIG. 4. Quantitative aerobic and anaerobic bacterial recovery (geometric means) from mucosal surface (A) and luminal contents (B) for the different treatments.

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