

Altering and Assessing Persistence of Genetically Modified *E. coli* MG1655 in the Large Bowel

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One of the primary factors limiting the efficacy of probiotic therapies is short persistence time. Utilizing a novel method for assessment of persistence in the large bowel independent of survival of the organisms in the upper GI tract, we tested whether overexpression of the type 1 pilus, a colonization factor, or the presence of secretory immunoglobulin A (slgA) might increase the persistence time of a laboratory strain of *E. coli* in the gut. For this purpose, cecal ostomies were created in mice and bacteria were placed in the ostomies, with or without slgA. The persistence of the bacteria was assessed by evaluating the length of time after placement in which the bacteria were found in fecal samples. *E. coli* MG1655 expressing pili with the mannose-specific adhesin persisted *in vivo* significantly longer [mean (hours) $\pm$ SEM: 91.50 ± 15.98 , $n = 12$] than bacteria expressing pili without adhesin [43.67 ± 8.22 , $n = 12$] ($P = 0.01$) and significantly longer than bacteria expressing neither pili nor adhesin [22.00 ± 4.22 , $n = 12$] ($P = 0.0004$). Although the persistence time of bacteria was not significantly affected by the presence of slgA, the slgA did cause a relative increase in retention of inert particles. These results, combined with an acute increase in stool production and stool water content in those animals not receiving slgA following introduction of bacteria, suggest that slgA might have anti-inflammatory properties in the gut when administered with enteric bacteria. Modifying expression of probiotic colonization factors may

provide substantial benefit to patients with digestive tract diseases by virtue of increased persistence of the probiotic and, in the case of slgA, an anti-inflammatory effect. This novel *in vivo* model may be useful in evaluating persistence time in a variety of current and future probiotic regimens. *Exp Biol Med* 234:1174–1185, 2009

Key words: probiotics; pilus; immunoglobulin A

Introduction

The interface between the human gut epithelium, the mucosal immune system, and commensal microorganisms has emerged as an extremely active frontier in medicine today. The link between the barrier function of the human gut and the numerous disease states resulting from an incompetent barrier is an area of considerable interest to the practicing clinician. Gram-negative bacteremia alone resulted in more than 150,000 bloodstream infections per annum from 1990 to 2000 (1). While mortality is decreasing in some patient subgroups, mortality continues to be 35–45% in severely septic patients, with the abdominopelvic region being one of the most common sources of pathogens (2). Inflammatory bowel disease, including Crohn's disease and ulcerative colitis, affects between 0.1% and 0.2% of the population and is increasing in developed countries. Each of these pathogenic states may be initiated at the boundary of the human gut epithelium and the intestinal microbiota.

Probiotics have been defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (3). In addition, it has been determined that specific strains are safe for human use and merit further scientific study (3). Emerging clinical trials have proven the effectiveness of probiotic use in a variety of conditions, including ileal pouchitis, postoperative infections, and inflammatory bowel disease, among others. The

This work was supported by the Fannie E. Rippel Foundation and by grant P30 DK34987 from the National Institutes of Health.

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Received December 4, 2008.
Accepted June 16, 2009.

DOI: 10.3181/0812-RM-358
1535-3702/09/23410-1174\$15.00
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rate of endoscopic recurrence of Crohn's disease after treatment was found to be lower in patients receiving antibiotics and probiotics than in patients receiving antibiotics alone (4, 5). Pouchitis, the most common long-term complication of total proctocolectomy with ileal pouch-anal anastomosis, has also been successfully treated with probiotics. In a double-blind study, a highly concentrated preparation of bacteria of eight different strains, VSL#3 (Sigma-Tau Pharmaceuticals, Gaithersburg, MD), was compared with placebo in 40 patients for efficacy in maintaining clinical and endoscopic remission of pouchitis. As measured by the Pouchitis Disease Activity Index, all 20 patients receiving placebo relapsed, while 17 out of 20 (85%) of the treatment group were maintained in remission at the termination of the study at 9 months. These 17 patients had relapse within 4 months of the termination of probiotic treatment (6).

The list of clinical scenarios in which probiotics might potentially be useful is extensive. Postoperative infections, for example, may be ameliorated by probiotics. Such infections occur in approximately 30% of patients after major abdominal surgery despite perioperative antibiotic use (7). Microorganisms found in the normal gut, including *Escherichia coli* (*E. coli*) and *Enterococcus* spp., are commonly isolated from surgical site infections and blood (8). Translocation from the intestinal lumen has been suggested as the source of these pathogens (9), with a significant association between postoperative morbidity and bacterial translocation. In a prospective, randomized trial, liver transplant patients given early enteral nutrition containing fiber-enriched formula plus live *Lactobacillus plantarum* 299 developed significantly fewer bacterial infections than did patients receiving early enteral nutrition with standard bowel decontamination or heat-killed *L. plantarum* 299 in fiber-enriched formula (10). In another study, early enteral supply of fiber and live *L. plantarum* decreased the incidence of postoperative infections, including sepsis, pneumonia, and peritonitis, among others. Of those who developed infections, patients in the *Lactobacillus* arm required antibiotic therapy for a significantly shorter period of time than did patients in the standard treatment or heat-inactivated *Lactobacillus* arms (7). Decreasing the incidence and duration of postoperative infections could translate into millions of health-care dollars saved. While selective gut decontamination may reduce translocation rates and septic morbidity, no firm evidence suggests an improvement in survival (9). Alteration of intestinal microflora with non-pathogenic strains may offer a safe alternative in susceptible individuals.

Despite numerous successes with probiotics in the clinical arena, research in this area is still in its infancy, with some studies showing little or no benefit to the use of probiotics, at least in some settings. For example, Chermesh et al. did not find that probiotics prevented the recurrence of Crohn's disease (11), and Marteau et al. (12) as well as Prantera et al. (13) also found no effect of probiotics on

prevention of recurrence of Crohn's disease. Further, in a study demonstrating clearly that probiotics might not be helpful in all situations, Besselink et al. (14) observed higher mortality rates in patients with severe acute pancreatitis as an apparent result of treatment with probiotics. Thus, much additional work is needed not only to test the efficacy of probiotics in various clinical settings, but also to improve the efficacy of probiotics.

Colonization factors, which likely play a key role in persistence, mediate bacterial adherence to both the host surface, including the human colonic epithelium, and to other microorganisms. Among a wide array of bacterial colonization factors, pili are non-flagellar proteinaceous appendages found in large numbers on approximately 70% of all fecal isolates of *E. coli* (15). The type 1 pili, or fimbriae, of *E. coli* are the most extensively described fimbrial system (16). Pili are subject to phase variation, in which individual cells can switch between piliated and non-piliated states. Colonization is often mediated by extremely selective adhesins binding to complimentary receptors on the target surface. In *E. coli*, around 1000 copies of the major subunit protein FimA serve as a scaffold for the FimH receptor-recognizing adhesin. The adhesin molecule interacts with mannose-containing moieties, which are present on virtually all eukaryotic cells (17), including mammalian epithelial cells. The adhesin on type 1 pili is located at the tip and possibly along the shaft of the FimA scaffold (16).

In this study, we demonstrate for the first time that constitutive expression of a colonization factor of *E. coli* improves the persistence time of the microorganism in the mammalian large intestine. In addition, we determined that the addition of secretory IgA (sIgA) apparently led to a reduced inflammatory response, as indicated by decreased stool production and decreased water content in the stool. These approaches can be applied to any bacteria, including commonly used probiotic strains of lactic acid-producing bacteria. More importantly, this work demonstrates that genetic modifications made with the intention of increasing persistence time in the large bowel can indeed successfully increase the duration of survival of selected microbes in the large bowel.

Materials and Methods

Study Design. In order to assess the possible protective effects of both bacteria and sIgA on the bowel, the physiologic response of the mouse colon to the introduction (via cecal ostomy) of bacteria in the presence or absence of sIgA was evaluated. The cecal ostomy was used as a model for two primary reasons: (a) The results are dependent only on the interactions of the bacteria with the large bowel and with the rest of the microbial flora. Variability that may be incurred in the stomach (e.g., varying degrees of cell death in the acidic environment) or in the small bowel is circumvented. (b) The direct insertion of probiotics into the large bowel is of substantial interest in

the clinical arena, particularly in patients undergoing surgery of the lower bowel.

In the first set of experiments, the persistence of three strains of *E. coli* in the colon was measured in the absence of IgA. Then, the effect of sIgA was evaluated using one strain. Inert latex microspheres were also introduced at the same time to provide a reference standard for bulk movement of material through the colon. *E. coli* strain MG1655 was utilized in this study because, although the organism is not utilized as a probiotic, the extensive characterization of this organism and the well established ability to genetically modify the organism make it ideally suited as a test case for establishing methodology for other strains that might be used as probiotics.

Stool samples were taken at regular intervals and weighed, cultured for bacteria, examined under light microscopy for presence of microspheres, and assessed for water content by weighing before and after freeze drying. The following parameters were compared for mice receiving bacteria + sIgA or bacteria alone: bacterial retention time, microsphere retention time, stool production in the first two hours and over 48 hours, and stool water content. A subset of mice was re-exposed to bacteria three additional times with a minimum of two weeks between exposures to investigate potential immune modulation of the parameters assessed.

Statistical analysis for all experiments was performed using the two-tailed Student's *t* test for unpaired samples, unless otherwise noted. All analyses were performed using Graphpad Prism® software, version 3.00, licensed to the author at Duke University.

Laboratory Mice. Forty-six female strain 129/SvEv mice were obtained from Taconic Farms (Germantown, NY). Before the mice were utilized in any experiments involving test bacteria, nine mice died or were sacrificed either because of complications from anesthesia during the surgical procedure or postoperatively due to infection. Two additional mice were excluded from the study due to a postoperative infection that responded to treatment with antibiotics. In total, 35 mice with patent mature ostomies were used in this study. All mice were housed in a containment facility at Duke University Medical Center, and all experiments were approved by the Duke University Institutional Animal Care and Use Committee.

Creation of Cecal Ostomy. In order to obtain access to the colon for experimental manipulation, a cecal ostomy was created surgically in 6- to 12-week-old strain Sv/Ev 129 mice, always within 4 weeks of receiving the mice. Mice were kept on a normal, ad lib diet from arrival through the night prior to surgery.

The mouse was anesthetized with 1.5–3% isoflurane in oxygen. The abdomen was shaved and the mouse was placed in the supine position on the operating surface. The operating surface included a dry diaper, electrocautery conduction plate, and insulated heating pad. The abdomen was prepped with Betadine and draped in a sterile fashion.

Under a microscope, the abdomen was entered through a 10–20 mm midline incision. A 4–5 mm circular opening was then made in the right lower quadrant (RLQ) using electrocautery. The blind end (apex) of the cecum was brought through this second opening and secured with a clamp. Warm normal saline (NS) was introduced into the peritoneal space for hydration and the midline incision was closed in two layers with non-absorbable 5–0 Prolene (Ethicon, Johnson & Johnson).

Through the RLQ opening, the cecal artery was identified and cauterized at the most distal visible bifurcation of the artery. At this level, approximately 1–2 mm of the cecum was excised and sewn to the musculature of the abdominal wall and the skin with interrupted 6–0 PDS (Ethicon). Care was taken that each stitch was placed through bowel wall, abdominal musculature, and skin to prevent fecal contamination of the abdomen.

Postoperatively, the animals received ampicillin 2 mg/kg/24 h for 72 h and buprenorphine 0.1 mg/kg every 12 h for 72 h (obtained from Sigma Chemical Company, St. Louis, MO, and Hospira Inc, Lake Forest, IL, respectively). All mice were monitored for distress according to the approved IACUC protocol. The non-absorbable Prolene sutures were removed 10 days after the procedure. Mice were not used in studies until at least 7 days after surgery to allow the bowel to return to baseline motility.

Complications that arose during the development of the procedure included intraoperative bleeding during ligation of the cecal artery and postoperative peritonitis presumably due to fecal contamination. These complications were avoided by ligating the cecal artery at the most distal aspect and delayed opening of the cecostomy to avoid fecal contamination of the open abdomen. Animals in distress in the perioperative period were euthanized according to an IACUC-approved protocol.

In order to ensure long-term tolerance of the procedure, distress and weight gain/maintenance of the initial group of mice in which cecal ostomies were created were monitored for six months. These mice were sacrificed at 12 months according to our protocol, while the other mice were sacrificed upon completion of the experiments. Over time, the ostomies in the first group of mice tended to close due to scarring. In fact, 11 of the 20 mice used in this group (52.3%) required revision of the ostomy, usually between 3 and 4 weeks after primary ostomy creation, to ensure patency for introduction of bacterial test strains. The revision consisted of mobilization of the ostomy, resection of 1–2 mm of the distal end of the cecum, and attachment of the remaining cecum to the abdominal musculature and skin.

Bacterial Strains and Growth Conditions. *E. coli* strains used in this study were derivatives of *E. coli* K-12 (see Table 1). All strains were propagated in L broth and L agar under conditions previously described (18) and were stored at –80°C in 10% glycerol prior to use. Piliated *E. coli* was detected in bacterial agglutination assays (BAG)

Table 1. *E. coli* Strains ORN 225–227 Were Resistant to Tetracycline Only (ORN225; pilus+), Tetracycline and Kanamycin (ORN226; pilus+, adhesin–), or Kanamycin Only (ORN227; pilus–)

Strains	Relevant properties	Source or reference
<i>E. coli</i> K-12		
MG1655	Original strain: F- λ -	38
ORN225	Stable expression of pili: MG1655 except <i>fimB-tetR</i> <i>fimE::IS1</i> Hag+ Bag+	19
ORN226	Stable expression of nonhemagglutinating type 1 pili: ORN225 except <i>fimH'-neoR</i> Hag- Bag+	19
ORN227	No expression of type 1 pili: MG1655 except Δ (<i>fimBEAICDFGH</i>)- Ω <i>neoR</i> Hag- Bag-	19

Hag+, capable of hemagglutinating guinea pig erythrocytes as described previously (19); Bag+, bacterial agglutination in polyclonal rabbit antiserum raised against purified type 1 pili as described previously (19).

that employed type 1 pilus-specific antiserum (18). Strains producing pili with a functional FimH adhesin were detected in hemagglutination (HAG) assays, using guinea pig erythrocytes as previously described (18).

Prior to evaluation of persistence time *in vivo*, *E. coli* were thawed and grown under microanaerobic conditions for 12–16 h at 37°C in Minimal Essential Medium Eagle (MEME; Sigma-Aldrich, Inc., St. Louis, MO) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1 mM Minimum Essential Medium (MEM) amino acids (Sigma-Aldrich, Inc., St. Louis, MO), and 1.0 mM sodium pyruvate (Invitrogen, Grand Island, NY). Low-salt LB broth media (Teknova, Hollister, CA) and Difco® agar (Becton, Dickinson and Company, Sparks, MD) were reconstituted, autoclaved, and cooled before adding antibiotic. Antibiotics were used in the bacteria detection plates at the following concentrations: tetracycline (40 µg/ml), kanamycin (40 µg/ml). Antibiotics were obtained from Sigma Chemical Company (St. Louis, MO) and Roche Diagnostics (Manheim, Germany), respectively, and dissolved in 0.15 M NaCl and sterile-filtered before being added to the molten sterile agar. Antibiotic agar was pre-poured into sterile Petri dishes (VWR International, Westchester, PA) and stored at 4°C until use. To ensure viability and antibiotic resistance of bacterial strains, 100 µl of each strain and fresh mouse stool in normal saline (10% w/v) were plated onto control plates from each batch of plates. Colony counts were assessed at 24 h.

Preparation of Bacterial Strains for In Vivo Experiments. In order to introduce the bacteria to the mouse colon in a controlled manner, bacterial samples were prepared as follows: Experimental strains of *E. coli* were grown as described above in the appropriate media and counted on a Coulter Multisizer II (Beckman Coulter, Fullerton, CA) with a 50 µm aperture. Based on the experimentally determined efficiency of the counter at detecting *E. coli* cells, the number of bacteria was calculated to be 100 times the number of counts obtained using the counter. A volume containing 1×10^9 bacteria/strain/mouse was delivered into a micro-centrifuge tube and spun at 10,000 rpm for 10 minutes. The bacterial pellet was

resuspended in 100 µl sterile 0.15 M NaCl, yielding a suspension of 1×10^{10} bacteria/ml.

Preparation of sIgA/Bacteria Mixture for In Vivo Experiments. Prior to introduction to the mouse colon, the bacterial suspension containing sIgA was prepared as follows: Human sIgA purified as previously described (19) was dialyzed in Spectra/Por molecularporous membrane bags against two changes of PBS, followed by a final dialysis overnight against normal saline. The solution was then sterile filtered using Acrodisc syringe filters.

Bacteria for experiments with sIgA were prepared by a slight modification of the method described above: MEM containing HEPES, non-essential amino acids, and sodium pyruvate were inoculated with *E. coli* strain ORN225 and cultured overnight in an incubator at 37°C. The number of bacteria was determined using the Multisizer, as above. 1×10^{10} bacteria were introduced into sterile eppendorf tubes and centrifuged to form a solid bacterial pellet. Supernatant was discarded and bacteria were resuspended in either 1 ml of normal saline or 1 ml of 0.5 mg/ml sIgA solution. Solutions were kept at room temperature 23°C for 35–50 minutes prior to introduction into cecal ostomy to allow sufficient time for sIgA agglutination of bacteria.

Preparation of Microspheres for In Vivo Experiments. The 10-µm latex beads (Polysciences Incorporated, Warrington, PA) were prepared for injection into the cecal ostomies. The retention time of these inert particles served as a reference standard for movement of bulk material through the colon. Ten drops (approximately 0.26 ml) of microsphere mixture (4.55×10^7 particles/ml) were added to 1 ml of normal saline in a sterile eppendorf tube. The mixture was centrifuged to pellet the microspheres. The supernatant was discarded and the spheres were washed with sterile normal saline and centrifuged again. Washing and subsequent centrifugation were repeated once more and spheres were resuspended in 50 µl of sterile saline prior to introduction into the cecal ostomies.

Introduction of Bacteria, sIgA, and Microspheres into Cecal Ostomies. The experimental animal was lightly anesthetized with 1.5–3% inhaled isoflurane in oxygen and placed in the supine position. The wall of the

cecal ostomy was grasped with blunt-tip forceps and lifted up to facilitate introduction of liquid material into the ostomy and minimize reflux.

In order to examine the persistence of various antibiotic-resistant strains of *E. coli* in the mouse colon, 1×10^9 bacteria/strain in 100 μ l 0.15 M NaCl were introduced into the cecal ostomy via a 24-gauge bulb-tip feeding needle fitted on the end of a 1-ml syringe. Mice were exposed to paired bacterial strains (two strains, simultaneously) to help determine whether variability between mice and between experimental trials was a source of variability for the overall results. Further, since multiple exposures of mice to the same bacteria were avoided (see Results section), this approach reduced the number of laboratory animals necessary for the study. Since all *E. coli* used in this study lack F-pili and are unable to conjugate, no inter-strain transfer of genetic material is expected. Stool samples were collected from each animal prior to introduction of bacteria through the ostomy and at various intervals following introduction of the bacteria. Following introduction of bacteria, stool was collected at 2-hour intervals for the first 12 hours, at 12-hour intervals the next 36 hours, and at 24-hour intervals thereafter. Samples were collected until no further antibiotic-resistant strains of *E. coli* were found for 48 hours. Bacterial persistence time was defined as the latest collection time point at which any exogenous bacteria grew on culture plates. Two successive negative culture results were required for confirmation that the bacteria no longer persisted.

In order to evaluate the potential of sIgA as an adjuvant for probiotic agents, prepared microspheres and ORN225 *E. coli* $\pm$ 0.5 mg/ml sIgA were also introduced into the cecal ostomy. Mice were then placed in clean cages and allowed to recover from anesthesia. Stool samples were collected and persistence time was determined as described above.

Bacterial Culture from Stool Samples. The amount of antibiotic-resistant *E. coli* present in stool samples was assessed as follows: Saline was added to the stool to make a 10% weight of stool/total volume stool mixture. Stool was physically broken up with a sterile pipet tip, followed by vortexing and sonication for 30 seconds. Stool was diluted as necessary to achieve colony separation and plated onto pre-poured antibiotic plates so that the strains might be differentiated by their antibiotic resistance genes. For example, ORN225 and ORN227 were resistant to tetracycline and kanamycin, respectively (see Table 1). Failure to include the antibiotics resulted in the growth of vast numbers of microorganisms present in the fecal flora, as was expected. Plates were placed in the incubator at 37°C and bacterial colonies were counted after 24 hours of incubation. The maximum amount of fecal material that could be reliably dispersed on a culture plate was determined to be 10 mg of stool diluted in a total volume of 100 μ l for the purpose of plating. Thus, the lower detection limit of one colony per plate corresponded to 100 CFUs per gram of fecal material. The persistence time was

measured as the last time point at which bacteria were found in the stool sample.

Stool Microsphere Assessment. To understand the physiologic effect of cecal ostomy on bulk movement through the mouse colon, passage of microspheres through the colon was assessed as follows: Saline was added to stool samples to make a 10% weight of stool/total volume mixture. Stool was physically broken up with a sterile pipet tip followed by vortexing. Stool was then filtered through a 70- μ m cell filter to remove bulk particles and allow isolation of microspheres. Filtered stool solution was placed on a hemacytometer and spheres were counted by visual inspection under light microscopy at $\times 100$ ($\times 10$ objective lens plus $\times 10$ ocular lens) magnification.

Stool Water Content Assessment. Fresh stool collected from mice was weighed, flash frozen in sterile specimen tubes, and stored pending analysis. At the time of analysis, specimen tubes were thawed, weighed, and then freeze-dried. Tubes were re-weighed afterward to determine the change in weight, attributed to evaporation of water from stool sample. Weighing the tubes again following removal of the dried material was utilized to assess the amount of dry material in the samples.

Results

Physiologic Changes Due to Cecal Ostomy.

The physiologic changes induced by surgical creation of cecal ostomies were examined. The first group of mice in which cecal ostomies were created was monitored for morbidity, mortality, and weight gain/maintenance for six months postoperatively. In addition, stool production and stool water content over a 48-hour period were compared in mice prior to surgery and 1–2 weeks after surgery.

The six-month postoperative survival, excluding perioperative death, of the mice used to test bacterial persistence was 96%—one postoperative death occurred outside of the perioperative period at two weeks from unknown causes. Mice surviving six months with mature ostomies tolerated the procedure well, as judged by lack of distress and normal weight gain/maintenance. No deaths were attributable to the revision procedure.

Average stool production was significantly increased in mice following surgical creation of an ostomy. Following at least a one-week recovery period following surgery, stool production was 0.078 ± 0.004 grams/hour ($N = 15$) after surgery versus 0.053 ± 0.002 grams/hour ($N = 21$) before surgery ($P < 0.0001$) (Fig. 1a). Surgery also increased the percent of water found in the stool samples by about 10%: Stool prior to surgery ($N = 22$) contained $63.1 \pm 1.2\%$ water, whereas stool after surgery ($N = 17$) contained $69.5 \pm 1.0\%$ water ($P = 0.0004$) (Fig. 1b).

The significant increases in stool production and water content following surgery are indicative of change in colonic water reabsorption from stool. These differences likely can be attributed to the alteration of bowel continuity

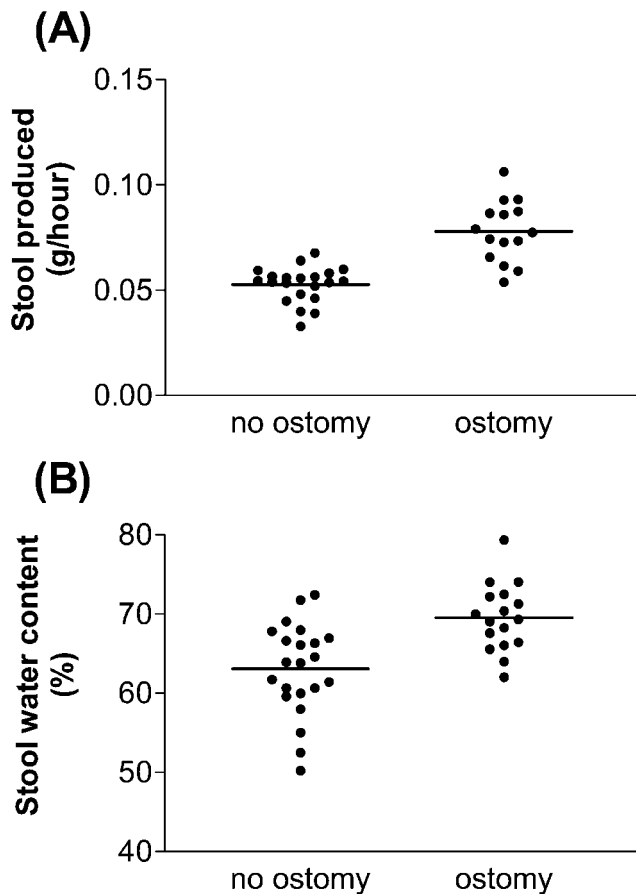


Figure 1. Stool production and water content were compared in mice before and after surgical creation of cecal ostomy. Both stool production and water content were significantly ($P < 0.0001$ and $= 0.0004$, respectively) increased following surgery, indicative of physiologic changes in colonic water reabsorption from stool.

by surgery and subsequent reduction in capacity of the cecum to reabsorb water from stool.

Role of Pili and Adhesin in the Persistence Time of *E. coli* K-12 In Vivo. A mutant (ORN225) of the *E. coli* K-12 strain MG1655, which constitutively expresses pili with adhesin, was evaluated along with a mutant of the same lineage which expresses pili without adhesin (ORN226), and a mutant that does not express pili (ORN227, see Table 1). As shown in Figure 2, MG1655 expressing pili with adhesin persisted significantly longer [mean (hours) $\pm$ SEM: 91.50 ± 15.98] than both MG1655 expressing pili without adhesin [43.67 ± 8.216] ($P = 0.01$) and MG1655 not expressing pili or adhesin [22.00 ± 4.221] ($P = 0.0004$). The comparison between MG1655 expressing pili without adhesin and MG1655 without pili was also statistically different ($P = 0.0284$), suggesting that the pilus construct without the adhesin confers some benefit of adherence *in vivo*.

These studies were performed on a group of ten mice that were exposed to paired bacterial strains (two strains, simultaneously) between one and three times, with no mouse being exposed to a single strain more than twice.

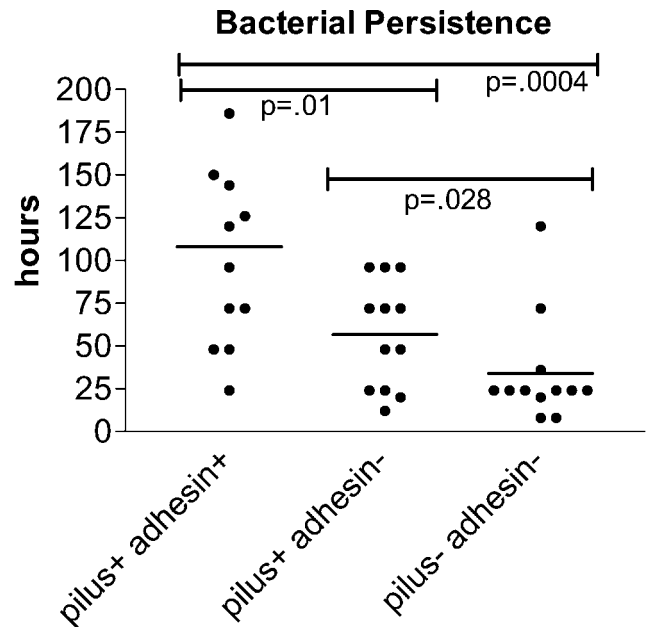


Figure 2. Role of pili in the persistence time of *E. coli* K-12 *in vivo*. MG1655 expressing pili with adhesin persisted significantly longer [mean (hours) $\pm$ SEM: 91.50 ± 15.98] than MG1655 expressing pili without adhesin [43.67 ± 8.216] ($P = 0.01$). In addition, MG1655 expressing pili with adhesin persisted significantly longer than the strain not expressing pili or adhesin [22.00 ± 4.221] ($P = 0.0004$). These studies were performed by introducing pairs of test strains into 20 mice. The comparison between MG1655 expressing pili without adhesin and MG1655 without pili suggested that expressing the incomplete pili conferred some benefit to adherence over no pili expression ($P = 0.0284$). Statistical analysis was performed using the Student's two-tailed *t* test.

Three of the ten mice were only exposed to one pair of strains. Four of the ten mice were exposed twice, and the remaining three mice were exposed three times. Subsequent exposures could not be correlated to the first exposure by the Pearson's product-moment correlation coefficient ($P = 0.895$), implying that persistence times were not mouse-specific. Since no mice were exposed to the same strain of bacteria more than twice, further experiments are needed to evaluate any decrease or increase in persistence with subsequent exposures.

Rate of Movement of *E. coli* K-12 Through the Large Bowel. To monitor the rate of bacterial movement through the colon, the concentration of bacteria (CFU/gram of stool) was monitored during the course of some of the experiments. The results from one trial are shown in Figure 3. The example shown in Figure 3 is representative of all experiments in that the vast majority of bacteria were flushed out of the colon within 24 hours, regardless of the strain of *E. coli* used. However, unlike persistence time, the number of colony forming units in the fecal material was a much less sensitive marker for the effects of the genetic modification of the bacteria than was persistence time. One apparent reason for this lack of sensitivity was that the rate at which bacteria were initially flushed through the colon, despite being rapid, was highly variable as assessed by our

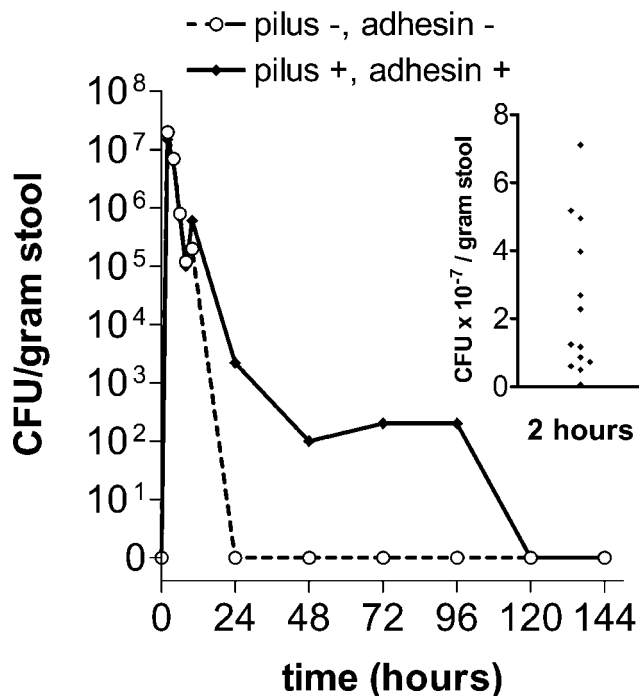


Figure 3. Movement of *E. coli* MG1655 through the large intestine following insertion through a cecal ostomy. *E. coli* laboratory strain MG1655 expressing pili with adhesin and the MG1655 strain that did not express pili were simultaneously introduced into the cecal ostomy of a 129-SvEv mouse. The amount of bacteria in the fecal material was then monitored over time, utilizing the antibiotic resistance markers to identify the different strains and distinguish them from the rest of the fecal flora. The results of one trial for the duration of the experiment are shown in the main graph. In this example, *E. coli* expressing pili with adhesin (indicated by the solid line) persisted in the mouse large intestine for 96 h, while *E. coli* without pili or adhesin (indicated by the dashed line, open circles) persisted for 12 hours. However, the vast majority of both strains were flushed out of the colon within 24 hours. In the inset graph, results from a number of trials following the presence of *E. coli* expressing pili with adhesin in the fecal material at 2 hours are shown. Of note is the extreme variation of bacterial counts in the stool at 2 hours post-inoculation.

methods. For example, in the inset of Figure 3, the CFUs of *E. coli* expressing pili plus adhesin in the fecal material at 2 hours post-inoculation from a number of trials are shown. Of note is the extreme variation of bacterial counts in the stool at 2 hours post-inoculation. This variation was observed throughout the time course of the experiments. For example, in 29 trials using *E. coli* expressing pili with adhesin, the number of bacteria persisting at the last time point at which the presence of bacteria was detected was 2986 ± 6355 CFUs/gram stool (mean $\pm$ SD), with a range from 100 CFUs/gram stool (the limit of detection of the assay) to 26,300 CFUs/gram stool (median = 500 CFUs/gram stool, coefficient of variation = 213%). Another factor which limited the use of CFUs in the fecal material as a sensitive measure (and probably accounted for some of the observed variability) was that all of the bacteria placed in the ostomy could not be accounted for in the fecal material in all experiments. One explanation may be that the sampling window was not sufficiently narrow to ensure

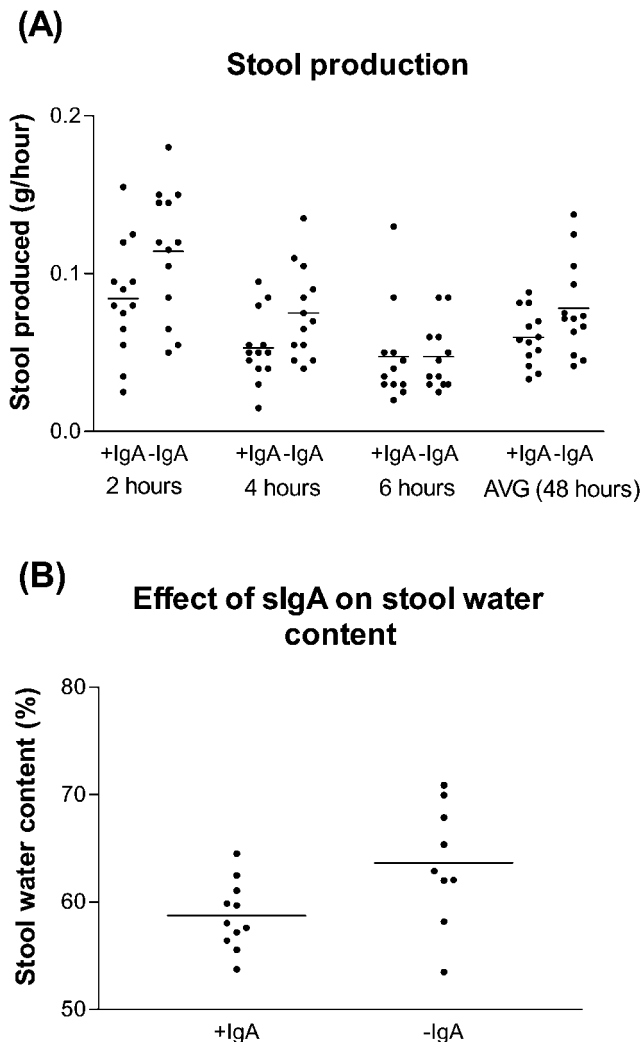


Figure 4. (A) Stool production in the 2–48 hours after introduction of exogenous *E. coli* into the cecum via ostomy was compared for animals receiving slgA vs. no slgA. Animals not given slgA produced greater amounts of stool in the first four hours ($P = 0.04$), but this difference disappeared by six hours and was not observed over 48 hours. (B) Stool water content in the first 48 hours after introduction of exogenous *E. coli* into the cecum via ostomy was compared for animals receiving slgA vs. no slgA. Water content appears to be increased ($P = 0.024$) in animals not receiving slgA.

that all bacteria were assessed. Another explanation for this may be aggregation of *E. coli*: Microscopic evaluation of the bacteria, particularly *E. coli* expressing pili with adhesin, revealed that the bacteria could auto-aggregate under some conditions, which would presumably reduce the number of CFUs measured, since each aggregate of bacteria only accounts for one CFU.

Effect of slgA on Stool Production. Stool production was measured over the first 48 hours following introduction of *E. coli* $\pm$ slgA into the cecum (Fig. 4a). Over this entire interval, there was no significant difference ($P = 0.43$) in stool production observed for mice receiving slgA (0.053 ± 0.003 grams/hour, $N = 9$) versus mice not receiving slgA (0.061 ± 0.01 grams/hour, $N = 9$). However,

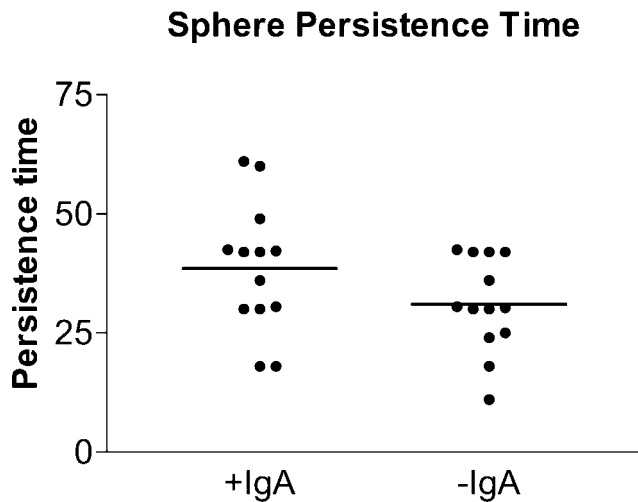


Figure 5. Microsphere persistence time was compared for animals receiving sIgA vs. no sIgA. Although spheres tended to persist longer in animals receiving sIgA, the trend was not statistically significant.

stool production in the first two hours exhibits a trend indicative of increased stool production in mice not receiving sIgA. This trend reached a significant difference at four hours, with mice receiving sIgA producing 0.053 ± 0.006 grams/hour ($N = 13$) and mice not receiving sIgA producing 0.075 ± 0.008 grams/hour ($N = 13$; $P = 0.04$). The effect is not seen at six hours (Fig. 4a). This pattern is consistent with an effect of sIgA concentration as it starts to increase soon after administration, peaks, then diminishes as time since administration increases.

Effect of sIgA on Stool Water Content and Microsphere Clearance. Stool water content was assessed by freeze-drying sample stool pellets taken every six hours for 48 hours after introduction of *E. coli* $\pm$ sIgA and measuring the change in sample weight (Fig. 4b). Stool water content was significantly lower in animals receiving sIgA ($58.74 \pm 0.95\%$, $N = 11$) compared to animals not receiving sIgA ($63.64 \pm 1.87\%$, $N = 9$) ($P = 0.024$).

The analyses of stool water content and stool production in the acute setting indicate that there is an increased amount of water present in the stool of the animals not receiving sIgA compared to those animals receiving sIgA. Two possible etiologies for this observation are: A) active secretion of water by the gut epithelium, as this has been shown to occur in rats (20), or B) decreased reabsorption of stool water by the gut epithelium, possibly due to either abnormally rapid passage of stool through the tract or an intrinsic decrease in absorptive capacity (21). These observations might suggest that the presence of sIgA ameliorates inflammatory responses that may be associated with the introduction of large numbers of bacteria into the gut.

The trend ($P = 0.12$) toward increased persistence time of microspheres in mice receiving sIgA offers further support for the idea that sIgA is ameliorating an inflammatory response. Spheres persisted in mice receiving sIgA for

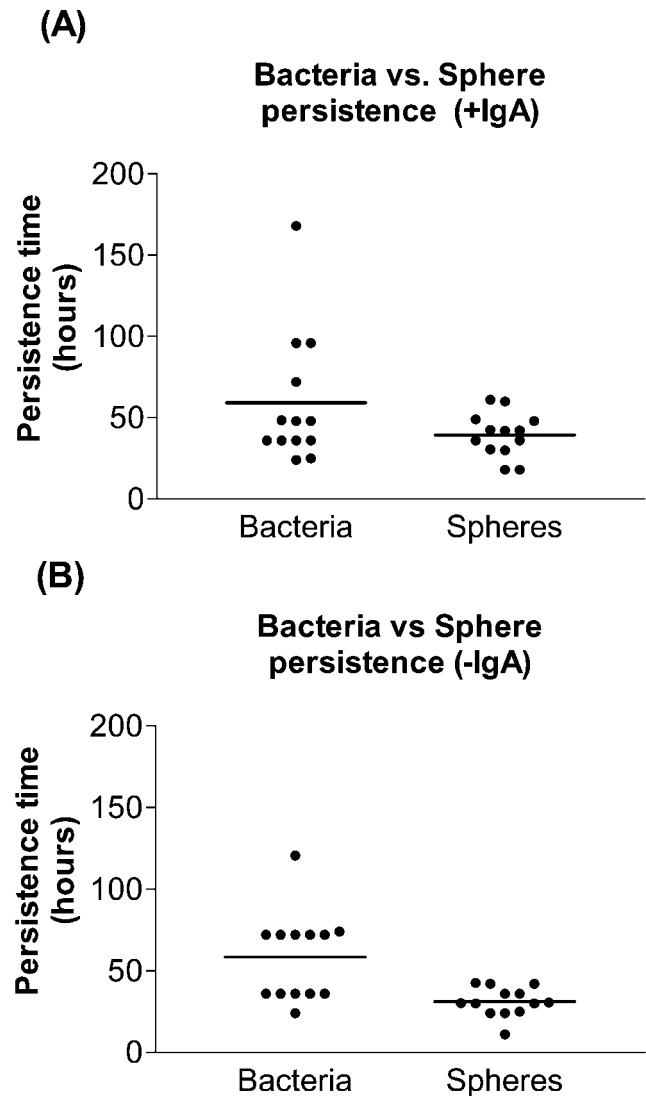


Figure 6. Persistence times of bacteria were compared to the persistence time of inert spheres in mice receiving sIgA and mice not receiving sIgA. Bacteria persisted in the gut longer than spheres; however, this difference was only significant in mice receiving sIgA. This observation is likely due to increased sphere persistence time in mice receiving sIgA.

38.56 ± 3.75 hours ($N = 13$) versus 31.02 ± 2.75 hours ($N = 13$; difference not statistically significant) in those mice not receiving sIgA (Fig. 5). However, the difference in persistence time between bacteria (results described below) and spheres was not statistically different in mice receiving sIgA ($P = 0.092$; paired t test), but was significantly different in those mice not receiving sIgA ($P = 0.006$; Fig. 6). The mean difference for mice receiving sIgA was 19.71 hours ($N = 13$), while the mean difference for mice not receiving sIgA was 27.33 hours ($N = 13$). This difference apparently reflects an increased sphere persistence time in mice receiving sIgA (Fig. 5).

Effect of sIgA on Bacterial Persistence. Bacterial persistence time was not apparently affected by sIgA. Persistence time for *E. coli* + sIgA was 59.19 ± 11.22 hours

($N = 13$) versus 58.35 ± 7.45 hours ($N = 13$) for *E. coli* alone ($P = 0.95$). The nearly identical bacterial persistence in the two groups of mice (those receiving sIgA and those not receiving sIgA) is potentially due to a combination of two factors. Given the trend toward increased microsphere persistence in mice receiving sIgA, it might be hypothesized that mice receiving sIgA would also show a trend toward increased bacterial persistence. However, this trend may be negated by sIgA-induced bacterial aggregation, which has been shown to occur *in vitro* under the conditions used in this study (19). Bacterial aggregation would presumably result in a decreased number of CFUs since the concentration of bacteria at long persistence times is frequently near the detection limit of the assay (see section above entitled, "Rate of Movement of *E. coli* K-12 Through the Large Bowel"), and clumping of bacteria would reduce the number of CFUs, possibly lowering the number below the detection limit in some cases. Thus, any potential increase in persistence time as a result of the presence of sIgA may be offset by the apparent decrease in persistence time caused by sIgA-induced bacterial aggregation.

Discussion

Improving *in vivo* persistence of a probiotic is of considerable interest. Adhesion of probiotic strains to target tissue epithelium and/or increasing the time of interaction with pathogenic strains may improve clinical efficacy. Various mechanisms have been proposed to describe the therapeutic effect of probiotics, all of which depend on the continued persistence of the bacteria in the bowel. Of the strains currently undergoing clinical testing, lactic acid producers such as *Lactobacillus* and *Bifidobacterium*, have been used in the most well-designed trials. For such bacteria that are often used as probiotic agents, a number of presumed anti-infective properties have been suggested. These include inhibiting the adhesion of pathogens (22), inhibiting the growth of pathogens (23), depleting nutrient resources, and creating an anti-inflammatory microenvironment (24, 25).

We used *E. coli* primarily as a test case for demonstration of principle. However, improving the persistence time of *E. coli* is not without some therapeutic interest, since *E. coli* has been proven effective as a probiotic in some studies. Corticosteroids given with oral gentamicin and non-pathogenic *E. coli* as a probiotic were shown to be as effective as corticosteroids with gentamicin and mesalazine in maintaining remission in patients with ulcerative colitis (26). Since patients with ulcerative colitis are more likely to carry pathogenic strains of *E. coli* (27), inducing a lasting change in colonic flora by removal of pathogenic bacteria and subsequent repopulation by a non-pathogenic strain of *E. coli* is a potential therapeutic target. Further, colonization of epithelial surfaces with non-pathogenic strains of *E. coli* may be beneficial in patients with a variety of pathologic states encountered by the

surgeon, including sepsis. Pathogenic strains, such as those causing septicemia or urinary tract infections, are more likely to belong to a group of *E. coli* which persist longer in the intestinal tract than are strains of other phylogenetic groups (28). Future studies may find a benefit of pre-operative probiotic loading of persistent, non-pathogenic strains. These strains would likely constitutively express type 1 fimbriae and may bind preferentially over pathogenic strains to receptor sites on the epithelium.

The importance of type 1 piliation in intestinal colonization by *E. coli* has been under considerable debate (29). The interaction between the type 1 pilus and receptor structures has been implicated to mediate *E. coli* colonization of host tissues (30), but *in vivo* studies of *E. coli* adherence to intestinal mucosa have drawn conflicting conclusions. The studies described herein provide the most direct evidence that the type 1 pilus mediates adherence of bacteria *in vivo*. These observations are consistent with other studies that indirectly implicate the type 1 pilus as being important *in vivo*: Several *in vitro* studies have established the interaction between the *E. coli* type 1 pilus and secretory IgA (sIgA) (19, 31). Work in our laboratory demonstrated that the formation of sIgA-mediated biofilms and mucin-mediated biofilms was dependent on the type 1 pilus (19), and formation of these biofilms may be key to bacterial persistence and even colonization (32, 33).

Using the same strains employed in this study, we previously found that our MG1655 *E. coli* strain expressing pili without adhesin (ORN227) can form sIgA-mediated biofilms on polystyrene, but not on live epithelial cells (34), while the MG1655 strain expressing pili and adhesin (ORN225) could form an IgA-mediated biofilm on polystyrene and on live epithelial cells. This evidence suggested that the adhesin may be important for binding to epithelial cell receptors and formed the basis of our hypothesis that the pilus would mediate increased persistence *in vivo*. Interestingly, our results *in vivo* more closely reflect *in vitro* results using polystyrene as a test surface than those using cultured epithelial cells as a test surface to evaluate adhesion of bacteria. This fact may reflect differences in the stringency of our *in vitro* assays or may indicate that binding to epithelium is only one pili-mediated interaction that affects persistence *in vivo*.

Adhesion of bacteria to surfaces or other microorganisms is mediated by numerous factors such as lipoteichoic acids, electrostatic interactions, and specific external structures (35). Little is known about the colonization factors of lactobacilli and other members of the indigenous microflora, aside from *E. coli*. *In vitro* evidence of a mannose-specific adherence mechanism has been found on *Lactobacillus plantarum* strains (36). This adhesive component has been suggested to be proteinaceous in nature, as treatment with proteinase K abolished the adherence of the organism. However, other strains have been associated with protease-resistant adhesion (37). Further characterization of the major adhesion-mediating substances is paramount if the

persistence of microbes in the gut is to be effectively manipulated. Genetically manipulating the promoter sequence of the gene for an adhesin-like protein in lactobacilli may enable increased, or even constitutive, expression of this bacterial structure. Increasing the concentration of this protein on the surface of the microbe may lead to increased adhesion in target tissues.

New strategies have been developed that use the cellular machinery of bacteria to combat disease. Recently, Rao et al. successfully colonized the mucous membranes of rats with a highly colonizing *E. coli* strain expressing an anti-HIV peptide (38). This peptide prevented viral transmission in high-exposure areas. In 2000, Steidler et al. reported that *Lactococcus lactis* that has been genetically modified to produce IL-10, an anti-inflammatory cytokine, has shown therapeutic promise in murine models of inflammatory bowel disease as well as patients with Crohn's disease (39). With the evidence that *E. coli* MG1655 constitutively expressing type 1 pili demonstrated significantly improved persistence, this strain may be engineered to over-express IL-10 to suppress inflammation. However, these strains must be carefully characterized in terms of translocation and permanent colonization.

Bacterial persistence in the gut is likely a complex function of several variables which can be roughly categorized as those promoting bacterial adhesion to gut epithelium versus those promoting extrusion from the gut. Variables that may promote adhesion include the degree of contact between bacteria and gut epithelium, the degree of bacterial expression of adhesion factors on cell surface, available nutrients at the interface, and the rate of incorporation into existing or new biofilms. Opposing these processes are variables promoting extrusion of bacteria from the gut which largely have to do with bulk movement of matter, including rate and strength of GI peristalsis, rate of stool production, stool consistency, and amount of food and water intake.

Of interest in this study were the substantial standard deviations associated with persistence times. The range of persistence time extended from less than 25% of the mean to more than double the mean, regardless of the bacterial construct used. This finding might reflect variation between individual mice, but the lack of consistently high or low persistence times in any particular mouse suggested otherwise. In addition, this variation was not due to intangible differences that changed from day to day, since experiments conducted simultaneously (within a given day) did not tend to be either high or low. One explanation for the large standard deviations is that the persistence time depends on factors within the bowel that fluctuate. For example, the amount of peristalsis at a particular time during our procedure may have a profound effect on the persistence time. The idea that persistence time depends on fluctuating variable(s) within the bowel, if correct, would have an impact on the methodology necessary to evaluate persistence time and on our understanding of factors underlying

persistence time. Perhaps more importantly, this idea may affect current insight into the therapeutic use of probiotics.

Developing effective probiotic regimens in the future will require evaluating properties that improve efficacy. Among the measures that may affect efficacy is persistence, or the time that the probiotic strain is present in the target intestinal segment following oral administration or inoculation. Increased persistence of bacteria in the large bowel is expected to improve the efficacy of any probiotic aimed at that region of the gastrointestinal tract. Although persistence of orally ingested bacteria in the gastrointestinal tract may be readily assessed, this persistence is often dramatically affected by survival of bacteria in the stomach and small bowel. Since probiotics may be administered through the anal route or through an ostomy, the actual persistence time in the large bowel is of interest as a factor independent of survival in the upper GI tract. Thus, assessment of bacterial persistence in the large bowel is of substantial importance.

In conclusion, the type 1 pilus with adhesin component expressed on all members of the population (ORN225) was found to increase the persistence time of *E. coli* in the murine large intestine. When this same strain was modified to express the type 1 pilus without the adhesin (ORN226), persistence was significantly decreased. Complete removal of the type 1 pilus (ORN227) resulted in still shorter persistence. With evidence that our method successfully differentiates persistence times of various strains of *E. coli* *in vivo* based on pili expression and construction, further studies are warranted to characterize the persistence of lactic acid producers, like *Lactobacillus* and *Bifidobacterium*.

The investigation of sIgA as a potential adjuvant for probiotic agents *in vivo* also offers evidence to support a promicrobial function of sIgA in the gut. While sIgA did not affect the persistence time of exogenous bacteria in a probiotic model as was initially hypothesized, the finding of no difference should not be regarded as trivial. One classic immunologic paradigm for sIgA is that the molecule is purely antimicrobial—that is, facilitating the rapid clearance of gut bacteria. If this were true, one would expect to observe significantly decreased bacterial persistence time in the presence of sIgA. On the contrary, persistence time was slightly increased in animals receiving sIgA, providing direct evidence that sIgA does not function purely in an antimicrobial fashion to clear bacteria from the gut.

As experiments were conducted, a possible anti-inflammatory role of sIgA emerged. In the acute setting following introduction of exogenous bacteria into the cecum, stool production and water content were increased in animals who did not receive sIgA with bacteria relative to animals receiving sIgA. Inflammatory changes in gut mucosa leading to either active secretion of water into the stool or reactive increase in gut motility with consequent decreased water reabsorption from the stool may be mechanisms contributing to this observation. Although the particular strain of *E. coli* used in experiments was intended to model a probiotic strain of bacteria and thus is not

pathogenic in humans, in a mouse model it is possible that some translocation of bacteria may occur to cause an inflammatory reaction. If this proposed mechanism of inflammation is true, the experimental observations suggest sIgA has a protective effect on gut mucosa, either preventing or attenuating inflammatory changes. However, direct tests of inflammation in the bowel during the course of future experiments will be necessary to evaluate this hypothesis.

The low virulence of the strain of *E. coli* used in this work limited a robust assessment of a potential anti-inflammatory role of sIgA. Some animals exposed to the bacteria seemed to exhibit an inflammatory reaction, but this was not uniformly seen. Utilizing a more pathogenic strain of bacteria to consistently cause inflammation in the mouse model would subsequently allow a more sensitive assessment of sIgA as an anti-inflammatory agent.

The authors wish to thank Mary Lou Everett for expert technical and logistical support.

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