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Evidence for Peptidoglycan Absorption in Rats with Experimental Small Bowel Bacterial Overgrowth

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Surgical creation of jejunal self-filling blind loops (SFBL) causes small bowel bacterial overgrowth which is associated with hepatobiliary inflammation in the susceptible Lewis and Wistar rat strains. Since hepatic injury occurs when small bowel anaerobic bacterial concentrations are increased 4 to 6 $\log_{10}$ units per ml and hepatic bacterial cultures are negative, we postulate that the inflammation is caused by absorption of phlogistic cell wall polymers originating from bacteria within the loop. To demonstrate absorption of bacterial cell wall polymers, we measured plasma and hepatic levels of immunoreactive peptidoglycan-polysaccharide (PG-PS) following intraluminal injection as well as anti-PG antibodies as an indirect measure of absorption and/or accumulation of endogenous PG. PG-PS purified from group A streptococci was detected in plasma by enzyme-linked immunosorbent assay after intraluminal injection; rats with SFBL showed significantly more uptake into plasma and the liver than sham-operated rats or SFBL rats which were treated with metronidazole ($P < 0.025$). Total plasma immunoglobulin A (IgA), IgG, and IgM levels did not differ among sham-operated rats and those with self-emptying blind loops or SFBL, but plasma anti-PG IgA ($P < 0.05$), IgG, and IgM ($P < 0.01$) levels were increased in rats with SFBL. Metronidazole and tetracycline prevented the elevation of anti-PG antibody, but gentamicin and polymyxin B did not. Anti-lipid A, anti-soy protein, and anti-chow antibodies in plasma were not consistently increased in rats with SFBL indicating the lack of a generalized antibody response to luminal antigens. These data suggest that PG from normal flora bacteria is absorbed from the intestinal lumen and that mucosal injury and/or increased luminal concentrations of PG, such as those induced by small bowel bacterial overgrowth, lead to enhanced absorption of potentially inflammatory bacterial polymers.

Bacterial cell wall structures, such as peptidoglycan-polysaccharide (PG-PS) polymers, have inflammatory and immunomodulating properties relevant to the pathogenesis of chronic inflammation (28). For example, a single intraperitoneal injection of PG-PS derived from group A streptococci (PG-APS) can cause chronic, spontaneously reactivating, erosive arthritis (4) and hepatic granulomas (30) in susceptible rat strains. Purified PG-PS derived from normal enteric floras can also induce chronic arthritis (24, 27) and granulomatous enterocolitis (23) in rats. We have postulated that PG-PS from normal enteric bacteria is involved in the pathogenesis of chronic inflammatory bowel disease and its associated systemic complications (13, 20, 23).

In a rat model of small bowel bacterial overgrowth (SBBO), SBBO develops within 1 week after surgical creation of the self-filling blind loop (SFBL) and causes intestinal mucosal injury, as indicated by the presence of disaccharidase deficiency (9, 25, 26), protein-losing enteropathy (10), and abnormalities of sodium and glucose transport (9, 25). Recently, we showed that susceptible rat strains develop hepatobiliary injury following the experimental induction of SBBO (12a, 13). Hepatic inflammation was characterized by portal-tract expansion with acute and chronic inflammatory cell infiltration and by areas of parenchymal inflammation with occasional necrosis, while biliary abnormalities consisted of bile duct proliferation, tortuosity of intrahepatic ducts, and dilatation of the extrahepatic ducts. Hepatic injury was not due to septicemia, peritonitis, bacterial infection of the liver, or nutritional deprivation (13). Hepatobiliary inflammation was, however, related to increased concentrations of anaerobic bacteria within the blind loop, since rats with self-emptying blind loops (SEBL), which have anaerobic bacterial concentrations 2 to 3 $\log_{10}$ units per ml lower than rats with SFBL, did not develop hepatic injury (13) and metronidazole prevented the lesions (14). SBBO may cause increased absorption of PG-PS because of increased luminal concentrations of bacterial cell wall polymers as well as enhanced mucosal permeability. Our hypothesis is that absorbed bacterial cell wall polymers play a role in the development of hepatic injury associated with SBBO (13). In this paper, we present evidence indicating that immunoreactive PG-PS can be absorbed from the intestine, on the basis of experiments in which plasma and hepatic PG-PS concentrations are measured following direct injection into the blind loop and on the basis of data showing an increase in plasma and luminal antibody response to endogenous PG after the creation of experimental SBBO.

This work was presented in part at the 11th International Convocation on Immunology, Buffalo, N.Y., June 1988, and at the annual meeting of the American Gastroenterological Association, New Orleans, La., May 1988.

MATERIALS AND METHODS

Animals. Inbred Lewis rats and outbred Wistar rats were used. Lewis rats were studied at 4 weeks postoperatively and Wistar rats were studied at 12 weeks postoperatively on the basis of the time course for the development of hepatic injury in these strains following the creation of SFBL (13). Initial body weights were 175 to 200 g, and weekly weights were recorded.

Surgical procedures. According to the method of Cameron et al. (3), 10 cm of jejunal SFBL was surgically created 7 cm

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distal to the ligament of Treitz by using a sterile technique and ether anesthesia. This method caused SBBO within 1 week following surgery (26). Control groups consisted of sham-operated animals which had laparotomies but no intestinal anastomoses and which did not develop SBBO, as well as rats which had SEBL in which the loops are constructed in an isoperistaltic fashion so that they continuously empty (13). Although SEBL have an increase in anaerobic bacteria of 1 to 2 $\log_{10}$ units per ml over baseline jejunal bacterial concentrations, manifestations of SBBO do not develop.

Experimental design. Heparinized plasma was collected from the tail vein immediately prior to surgery and then 2 weeks after surgery for Lewis rats and 2 and 6 weeks after surgery for Wistar rats. After the rats were killed by cervical dislocation, blood, peritoneums, abdominal organs, and intestinal loop contents were anaerobically cultured as described below, and intestinal mucosas from the loops were harvested for disaccharidase analysis. Luminal contents of the ceca and blind loops or the corresponding 10-cm segments of jejunum of sham-operated rats were flushed with 10 ml of iced saline and stored for immunoglobulin determinations at −20°C in the presence of aprotinin, phenylmethylsulfonyl fluoride, and azide as previously described (15). Intestinal segments were fixed in 10% buffered Formalin and then processed for histology and stained by using hematoxylin and eosin and periodic acid-Schiff stain diastase digestion techniques.

Antibiotic treatment. Within 24 h of surgery, rats were allowed to drink ad libitum. The daily fluid volume consumed was recorded, and antibiotic was added so that the appropriate dose was ingested. Since each rat consumed 25 to 30 ml of water daily, oral antibiotics were added to a 50-ml volume such that the total daily dose required was contained in 25 ml. The fluid volumes consumed by the rats did not change after addition of antibiotics. The excess solution was discarded daily. Oral doses of antibiotics were administered as follows (in milligrams per kilogram of body weight per day): gentamicin, 30; polymyxin B, 9; metronidazole, 50; and tetracycline, 20. While testing intraperitoneal injections of polymyxin B, we observed 50% mortality within 1 to 2 h due to respiratory arrest with doses recommended by Nolan and Leibowitz (19) and Gut et al. (8). A nonlethal dose of 0.5 mg/kg/day given intraperitoneally was determined by titration.

Measurement of total IgA, IgG, and IgM. Antibodies were measured by enzyme-linked immunosorbent assay (ELISA), using a sandwich technique as described previously (15). All dilutions were in phosphate-buffered saline (PBS), pH 7.4, such that 100 μl was applied to each well. Microtiter plates (Dynatech, McLean, Va.) were washed three times with PBS-Tween (0.5%) between each incubation step. Microtiter wells were coated with 500 ng of affinity-purified anti-rat immunoglobulin A (IgA), IgG, or IgM antibody for 15 h at 4°C. After unbound sites were blocked with 0.5% bovine serum albumin, samples or immunoglobulin standards were incubated for 8 h at 25°C. Subsequently, affinity-purified anti-rat IgA, IgG, or IgM antibody which was conjugated to alkaline phosphatase (AP) was applied for 15 h at 4°C. Color developed in the presence of a *para*-nitrophenyl substrate (Sigma tablets [5 mg/ml]; Sigma Chemical Co., St. Louis, Mo.), and the results were read with an ELISA reader at an optical density of 410 nm after 30 min. An IgA standard was prepared from purified rat bile (15). Anti-rat IgA was affinity purified from goat anti-rat secretory IgA (ICN Immunobiologicals, Lisle, Ill.) by using affinity chromatography with purified rat biliary IgA bound to Reactigel (Pierce Chemical Co., Rockford, Ill.) and conjugated with AP as described previously (15). The following were purchased: rat IgM standard (Calbiochem, La Jolla, Calif.); affinity-purified rabbit anti-rat IgG-AP (Sigma Chemical Co.) and rat IgG standard, affinity-purified rabbit anti-rat IgG, rabbit anti-rat IgM, and rabbit anti-rat IgM-AP (Cappel, Organon Teknika, West Chester, Pa.). Standard curves were linear in the following ranges: IgA, 0 to 100 ng/ml; IgG, 0 to 200 ng/ml; and IgM, 0 to 5 μg/ml. The coefficient of variance was less than 10% for each assay.

Antigens used in the ELISA. PG for the initial coating of the solid-phase ELISA plate was purified from *Streptococcus pyogenes* D58 as previously described (6). Briefly, bacteria were fragmented in a Braun shaker, cell walls were recovered by sodium dodecyl sulfate treatment, and then PG was extracted with hot formamide. Lipid A was purchased from Ribi Immunologic and Chemical Research (Hamilton, Mont.). Soy protein was a generous gift from Wesley Burke, University of Arkansas at Little Rock. A mixed "chow antigen" was made by dissolving 30 g of rat chow (Agway Inc., Syracuse, N.Y.) in 100 ml of water and then homogenizing and centrifuging the solution so that the supernatant was decanted with a final protein concentration of 5 mg/ml.

Measurement of antibody. In the first step of the ELISA for anti-PG antibody, microtiter wells were coated with PG (2 μg/ml diluted in PBS) and incubated for 15 h at 4°C. The remaining steps were identical to those described above, with the same affinity-purified anti-rat IgA, IgG, and IgM-AP conjugates used. The standard curves for each immunoglobulin were in a lower range because the amount of specific antibody is less than the total immunoglobulin (IgA, 0 to 15 ng/ml; IgG, 0 to 25 ng/ml; and IgM, 0 to 500 ng/ml). The time for color reaction of these assays was 90 min. Preincubation of plasma with 10 to 50 ng of PG per ml resulted in appropriately lower measurements of specific antibodies of all three immunoglobulin subclasses. For lipid A, soy protein and mixed chow antigen (2 μg/ml in PBS) were used as the initial coat on the Dynatech microtiter plates.

Bacterial cultures of organ specimens. A modification of the technique of Berg was used (1). The abdomen was shaved and then washed with Pharmadine (Sherwood Pharmaceuticals, Mahwah, N.J.). The skin was opened with one set of sterile instruments, and then the muscle and peritoneum were opened with a second set of sterile instruments. Peritoneal cultures were performed by swabbing the viscera with a sterile cotton-tipped applicator stick. Specimens of the liver, spleen, and mesenteric lymph node (MLN) were excised with a separate set of sterile instruments, weighed in sterile Kimax brand Ten Broeck tissue grinders (American Scientific Products, McGraw Park, (Ill.), and homogenized by using 0.2 g of tissue per ml of brain heart infusion (BHI) (Scott Laboratories, Inc., Fiskeville, R.I.) for MLN specimens and 1.0 g of tissue per ml of BHI for liver and spleen specimens. Cardiac blood was aspirated in a sterile fashion. All specimens were inoculated on prereduced BHI agar plates which were cultured anaerobically in BBL GasPak jars (Becton Dickinson, Cockeysville, Md.) at 37°C for at least 72 h.

Bacterial cultures of intestinal contents. Aliquots of intestinal fluid for antibody determination were collected prior to the addition of inhibitors, homogenized, diluted serially in BHI from 10^{-1} to 10^{-10}, and cultured anaerobically to determine bacterial concentrations of the loop or jejunum.

Measurement of disaccharidases. Intestinal mucosa was removed by being scraped with a glass slide while on ice, weighed, and homogenized in 5 mM EDTA with a Waring

blender. The homogenate was stored at −20°C in the presence of azide (0.02%), aprotinin (500 kIU/ml), and phenylmethylsulfonyl fluoride (0.5 mM). Mucosal disaccharidases (lactase and sucrase) were determined by the method of Dahlqvist (5).

Measurement of proteins. Plasma and mucosal proteins were measured by the method of Lowry et al. (16).

Intraluminal injection of PG-PS. In order to show that PG-PS could be absorbed from the intestinal lumen, Lewis rats were injected intraluminally with PG-PS (15 μg of rhamnose per g of body weight) 4 weeks following SFBL surgery. Sham-operated and metronidazole-treated SFBL groups of rats were also used. PG-PS polymers were purified from *S. pyogenes* D58 (group A) and sonicated as previously described to yield fragments ranging from 5×10^6 to 5×10^8 Da (22, 23, 27). At 4 weeks, rats underwent laparotomies, and the blind loops or corresponding sham-operated jejunal segments were injected via a 24-gauge catheter (Jelko IV Catheter; Critikon Inc., Tampa, Fla.) and then flushed with 1 ml of normal saline to prevent peritoneal soiling. The catheter was removed immediately after injection. Tail vein plasma was obtained prior to injection and 2, 7, 10, 16, 19, 22, 25, 28, 31, and 42 h later. At the 42-h sampling, when the rats were killed, portal-vein blood was obtained in addition to tail vein blood.

Measurement of PG-APS in plasma and liver homogenates. By using an affinity-purified antibody to group A streptococcal polysaccharide, PG-APS was measured by an ELISA technique as previously described (22). Liver tissue was homogenized in a Tris buffer with 1% Tween 20, pH 7.4, in a ratio of 4 ml of buffer to 1 g of tissue.

Statistical analysis. Antibody and disaccharidase levels for the experimental groups were compared by using the two-tailed Student's *t* test. The incidences of positive bacterial cultures of MLNs were compared by using the chi-square method.

RESULTS

Luminal bacterial counts. There was an increase in anaerobic bacteria of 0.7 to 1.7 $\log_{10}$ units per ml in rats with SEBL compared with the amount in sham-operated rats, but rats with SFBL had an increase in luminal anaerobic bacteria of 3.5 to 3.6 $\log_{10}$ units per ml compared with the number in rats with SEBL (Table 1). There was no significant difference between the totals of anaerobic bacteria in SFBL rats of the Lewis and Wistar strains.

Measurement of systemic absorption of PG-PS injected into the intestinal lumen. We first sought to determine whether immunoreactive PG-PS injected into the proximal small bowel would be systemically absorbed. Following injection of purified PG-APS into the SFBL or comparative sham-operated jejunal segment, blood samples were collected and tested by ELISA for PG-APS immunoreactivity. The time course is shown in Fig. 1, which presents the mean plasma value at each time point for the three rat groups. The areas under the curves represent total plasma uptakes, which are compared in Table 2 for sham-operated rats, SFBL rats without antibiotic treatment, and SFBL rats with metronidazole treatment. All rats had PG-APS present in plasma, generally peaking 7 to 10 h after injection. The area under the curve was significantly greater for untreated rats with SFBL than for those treated with metronidazole ($P < 0.025$) or sham-operated rats ($P < 0.020$). The area under the curve for the metronidazole-treated rats with SFBL was not significantly different from that for sham-operated rats. For all groups of rats, values for portal-venous blood drawn at 42 h were each higher than the values for tail vein blood drawn simultaneously, indicating absorption of PG-APS by the intestine, with subsequent hepatic extraction and/or dilution by systemic blood. Finally, liver PG-APS accumulation 42 h after luminal injection was markedly increased in SFBL rats which received no antibiotic therapy compared with that in SFBL rats treated with metronidazole and sham-operated rats. Levels of PG-APS in liver homogenates from metronidazole-treated rats with SFBL were no different from levels for sham-operated rats. Plasma and liver homogenates from 10 Lewis SFBL rats which were not injected with PG-APS were tested by the ELISA. The plasma samples ranged from 0 to 0.5 ng/ml, and the liver homogenates ranged from 0.5 to 2.0 ng/g of liver. These values are slightly higher than the background of buffer in the ELISA and approximately 10 times lower than the lowest values obtained for rats injected with PG-APS.

TABLE 1. Weight gain and bacterial counts in rats with and without blind loops

Experimental group[a]	*n*	Wt gain (in g)[b]	Log_{10} bacteria (CFU/ml)[b]	No. of rats with culture-positive MLNs[c]/no. tested
Lewis				
SHAM	8	41 ± 10	3.9 ± 0.6	1/5
SEBL	5	36 ± 18	5.6 ± 0.4[d]	2/5
SFBL	8	10 ± 15[e]	9.1 ± 0.5[d]	6/7
SFBL Gent	10	24 ± 15[e]	8.5 ± 0.5[d]	6/10
SFBL Poly	10	−20 ± 19[d]	9.2 ± 0.9[d]	10/10
SFBL Met	7	44 ± 25	8.3 ± 0.4[d]	5/6
SFBL Tetra	7	30 ± 12	9.1 ± 0.2[d]	5/7
Wistar				
SHAM	10	324 ± 28	4.9 ± 0.6	2/6
SEBL	4	271 ± 42[d]	5.6 ± 1.2	0/3
SFBL	11	209 ± 74[d]	9.2 ± 0.7[d]	6/7
SFBL Met	7	239 ± 69[d]	8.3 ± 0.9[d]	5/6

[a] SHAM, Sham operated; Gent, treated with gentamicin; Poly, treated with polymyxin B (pooled group treated with either oral or intraperitoneal injections); Met, metronidazole; Tetra, tetracycline. Lewis rats and Wistar rats were tested at 4 and 12 weeks after surgery, respectively.
[b] All values are mean ± SD.
[c] Number of rats with MLNs which grew bacteria when cultured.
[d] $P < 0.001$ versus sham-operated rats.
[e] $P < 0.05$ versus sham-operated rats.

Antibody responses to endogenous PG-PS. To determine whether an immune response to proliferation of bacteria within the SFBL developed, we measured plasma and luminal anti-PG antibodies.

Total immunoglobulin levels in plasma. Plasma immunoglobulin levels in Lewis and Wistar rats showed no change in total IgG or IgM at 4 and 12 weeks, respectively, after the creation of SFBL, SEBL or sham operation. Wistar and Lewis rats with SFBL had mildly increased plasma IgA levels, 17 and 61%, respectively, compared with levels for non-SFBL rats ($P < 0.05$). Antibiotic treatment did not alter total antibody concentrations.

Plasma levels of antibodies to PG and luminal antigens. Compared with sham-operated rats, Lewis rats with SFBL for 4 weeks and Wistar rats with SFBL for 12 weeks developed significant increases in specific anti-PG antibody for all three immunoglobulin classes in plasma (Table 3) despite the fact that total immunoglobulin levels were not different among the groups. The increased anti-PG antibody

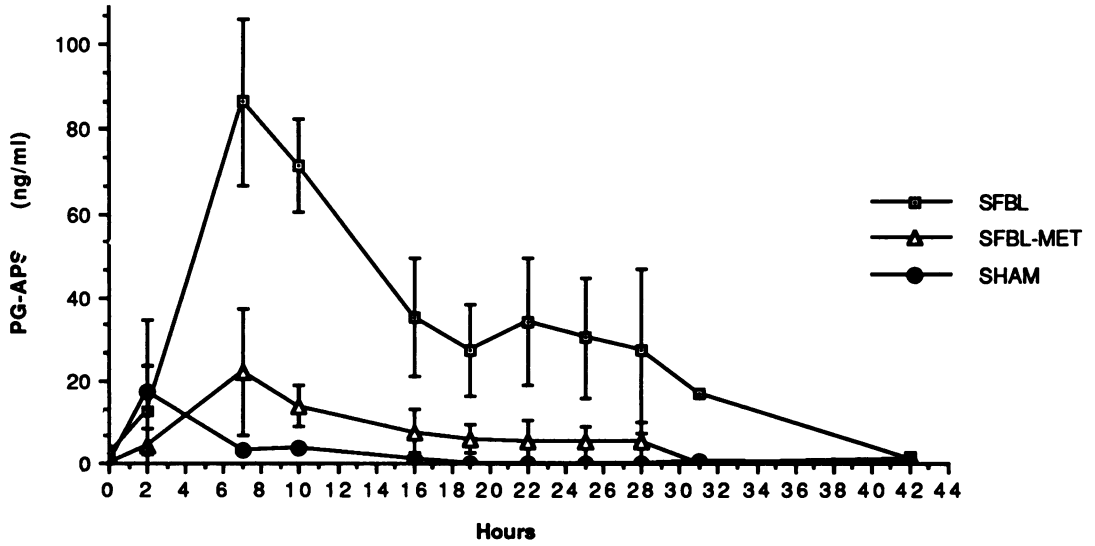

FIG. 1. Plasma PG-APS levels following intraluminal injection of PG-APS. The curves represent the mean ± standard error for plasma PG-APS for the three rat groups at each time point. PG-APS (15 μg/g of body weight) was injected intraluminally into the SFBL or the corresponding jejunal segments of sham-operated Lewis rats 4 weeks after surgery. Plasma PG-APS was measured by ELISA with affinity-purified antibodies to group A streptococcal polysaccharide. SFBL-MET, Rats which had SFBL and which were treated with metronidazole.

levels could not be attributed to surgical manipulation of the intestine, since rats with SEBL did not have elevated levels compared with those of sham-operated rats. The rise in anti-PG antibodies was not altered in rats treated with gentamicin (except for anti-PG IgA) or polymyxin B but was prevented by metronidazole and tetracycline. Rats which had SFBL and which were treated with metronidazole and tetracycline had significantly lower anti-PG antibody levels than SFBL rats which received no antibiotic treatment. There was a small but statistically significant rise in anti-PG IgG and anti-PG IgM levels in Lewis rats and in anti-PG IgM in Wistar rats with sham operations at 4 and 12 weeks, respectively, following surgery. However, the elevated anti-PG IgG and anti-PG IgM levels in SFBL rats which had no antibiotic treatment, which received gentamicin, or which received polymyxin B treatment were significantly greater than the naturally elevated levels found in the sham-operated rats (Fig. 2 and Table 3).

The rise in anti-PG IgA found in Lewis and Wistar rats with SFBL was not explained solely by the increase in total IgA. The percentage of total plasma IgA which was specific anti-PG IgA increased from 0.16 to 0.72% (450% increase) in Lewis rats and from 0.18 to 0.83% (450% increase) in Wistar rats, compared with only a 61% increase in total IgA in Wistar rats and a 17% rise in total IgA in Lewis rats.

For Lewis and Wistar SFBL rats with no antibiotic treatment, plasma anti-PG antibody levels showed an increase with time (Fig. 2). The levels were significantly greater than preoperation levels at 4 weeks in Lewis rats and at 6 and 12 weeks in Wistar rats.

Using similar ELISA techniques on the same blood samples as those tested for anti-PG antibody, we were unable to show consistent elevations of specific anti-lipid A, anti-soy protein, or anti-whole chow antibodies in the same groups of Lewis rats for which data are shown in Table 3 (data not shown). Sporadic increases in antibodies occurred; for example, anti-soy IgM levels increased 50%, anti-chow IgM levels increased 30% in rats with SFBL, and anti-lipid A IgG levels increased 20% in rats treated with tetracycline (for all, $P < 0.05$), but these elevations are of lower magnitude than and are not as consistent as the elevations of anti-PG antibodies shown in Table 3. These sporadic increases represent only 3 elevations of 45 data (3 antigens, 3 immunoglobulins, and 5 groups of Lewis rats [with no antibiotic treatment or with gentamicin, polymyxin B, metronidazole, or tetracycline treatment]).

Intestinal IgA levels. Wistar rats had more total IgA within the blind loop than did Lewis rats (Table 4) because their loops were larger because of greater body weight. Control 10-cm jejunal segments from sham-operated rats or SEBL rats had significantly lower concentrations of luminal IgA than did blind loops, by a factor of approximately 30, as previously reported (18). Because of the large difference in IgA in jejunal segments of SFBL rats and sham-operated or SEBL rats, we also studied IgA from the ceca and discovered that total cecal IgA in Wistar and Lewis rats was not significantly different among rats with SFBL, SEBL, or sham operations (Table 4). The blind loops contained more total IgA than ceca, but this difference was also due to larger volumes, since the concentrations (approximately 300 μg/

TABLE 2. PG-APS absorption following intraluminal injection[a]

Experimental group[b]	Area (cm^2)[c]	Amt of PG-APS in specimen from:		
		Liver (ng/g)	Portal vein (ng/ml)	Tail vein (ng/ml)
SHAM	5.5 ± 4.7	30 ± 19	7.0 ± 12	0.5 ± 1.2
SFBL Met	8.5 ± 6.7	46 ± 7	5.6 ± 5.0	1.3 ± 2.3
SFBL	62.0 ± 24.0[d]	262 ± 45[e]	12.7 ± 7.0	1.0 ± 1.7

[a] All values are mean ± SD.
[b] For each group, $n = 3$. SHAM, Sham-operated rats; Met, metronizadole.
[c] Area refers to the area under the curve shown in Fig. 1. The area reflects total PG-APS plasma uptake over 42 h.
[d] $P < 0.025$ for SFBL rats which received no antibiotic treatment compared with metronidazole-treated SFBL and sham-operated rats.
[e] $P < 0.001$ for SFBL rats which received no antibiotic treatment compared with metronidazole-treated SFBL and sham-operated rats.

TABLE 3. Plasma anti-PG antibody in rats with and without blind loops

Experimental groups[a]	n	ng of antibody/ml (mean ± SD)		
		IgA	IgG	IgM
Lewis				
Preop	18	311 ± 51	1,348 ± 651[b]	1,119 ± 540[b]
Postop				
SHAM	5	310 ± 48	2,461 ± 978	1,586 ± 255
SEBL	5	414 ± 80	3,842 ± 2,605	1,876 ± 351
SFBL	8	1,915 ± 819[c]	4,137 ± 1,836[b]	2,491 ± 789
SFBL Gent	10	382 ± 64	3,508 ± 824[b]	2,879 ± 966[b]
SFBL Poly, P.O.	6	747 ± 214[c]	4,869 ± 2,052[b]	3,967 ± 1,390[c]
SFBL Poly, I.P.	4	1,186 ± 324[c]	4,472 ± 1,651[b]	4,121 ± 1,575[d]
SFBL Met	7	302 ± 49[e]	2,574 ± 432[f]	1,237 ± 367[f]
SFBL Tetra	7	316 ± 106[e]	2,649 ± 570	1,361 ± 341[f]
Wistar				
Preop	10	420 ± 350	1,282 ± 809[c]	837 ± 441
Postop				
SHAM	9	480 ± 410	3,251 ± 1,571	1,070 ± 373
SEBL	4	371 ± 107	1,478 ± 665[d]	781 ± 412
SFBL	9	2,690 ± 2,020[d]	10,306 ± 4,730[d]	5,115 ± 826[c]
SFBL Met	7	295 ± 25[f]	2,398 ± 1,488[e]	967 ± 215[e]

[a] Lewis rats and Wistar rats were tested at 4 and 12 weeks after surgery, respectively. Preop, Blood drawn before surgery; Postop, blood drawn at the end of the experiment; p.o., per os; i.p., intraperitoneally. For other abbreviations, see Table 1, footnote *a*.
[b] $P < 0.05$ compared with sham-operated rats.
[c] $P < 0.005$ compared with sham-operated rats.
[d] $P < 0.01$ compared with sham-operated rats.
[e] $P < 0.001$ compared with SFBL rats which received no antibiotic treatment.
[f] $P < 0.05$ compared with SFBL rats which received no antibiotic treatment.

ml) were not significantly different among the experimental groups.

Intestinal anti-PG IgA levels. Total specific anti-PG antibody levels in loops of Lewis and Wistar rats with SFBL (378 and 1,172 ng, respectively) were significantly higher than in control 10-cm jejunal segments which contained 8 and 5 ng, respectively, of specific antibody (Table 4). SFBL Lewis and Wistar rats which were treated with metronidazole had significantly lower intestinal anti-PG IgA levels than those which received no antibiotics ($P < 0.005$ and $P < 0.05$, respectively).

Lewis rats with SFBL for 4 weeks and Wistar rats with SFBL for 12 weeks also had significantly increased specific cecal anti-PG antibody levels compared with those of sham-operated and SEBL groups (Table 4), by approximately twofold. This increase in specific cecal anti-PG antibodies was prevented by metronidazole treatment. Total specific cecal antibody levels in the sham-operated and SEBL groups were not different for the two strains.

Evidence of intestinal injury. To demonstrate the presence of intestinal mucosal injury, which could permit enhanced luminal PG absorption (20), we compared jejunal lactase and sucrase in rats with and without blind loops (data not shown). Following construction of SFBL, both Lewis and Wistar rats showed a significant decrease (approximately 50%) in disaccharidase levels. Metronidazole and tetracycline each prevented disaccharidase deficiency in SFBL rats, but gentamicin and polymyxin B did not. Rats which had SFBL and which were treated with metronidazole and tetracycline had higher disaccharidase levels than SFBL rats which were not treated with antibiotics (significance, $P < 0.05$ [except for lactase levels in Lewis rats treated with metronidazole]).

We compared the histologies of intestinal blind loop mucosas from nine Lewis rats and nine Wistar rats. Under light microscopy, all specimens demonstrated the findings typical of SBBO reported elsewhere (18, 29), including intact epithelial layers with no ulcerations. There was marked hypertrophy of the muscle layers. All specimens had an increased number of lymphocytes within the lamina propria, and some specimens had polymorphonuclear leukocytes. There were no consistent histologic differences between the rat strains.

Relationship of MLN culture to antibody response. In this study, none of the rats with SFBL had infection of the blood, peritoneum, liver, or spleen to account for the elevated plasma antibody levels. However, 86% (12 of 14) of SFBL rats given no antibiotic treatment had positive cultures of the MLN, compared with only 26% (5 of 19) of sham-operated and SEBL rats (chi-square = 11.45; $P < 0.01$) (Table 1). Positive MLN cultures occurred in 80% (31 of 39) of SFBL rats which received antibiotic treatment. Even tetracycline and metronidazole failed to prevent positive MLN cultures, since 79% (15 of 19) were positive. These frequencies of positive MLN cultures were not statistically different from those for SFBL rats which received no antibiotic treatment. To determine whether positive MLN cultures would cause elevated plasma anti-PG antibody levels, these variables were correlated. For the 33 rats with no antibiotic therapy (14 SFBL, 8 SEBL, and 11 sham operated), there was no correlation of a positive or negative MLN culture with antibody levels. Since each rat had plasma anti-PG IgA, IgG, and IgM levels, there were 99 possible matched pairs (i.e., positive cultures with elevated antibody levels or negative cultures with no change in antibody level). Of the 99 total possible matched pairs, only 52 actually matched (47 did not match), with no difference noted between rat strains or among antibody classes. Therefore, increased plasma antibody levels were not explained by translocation of viable organisms from the intestinal lumen to the MLN.

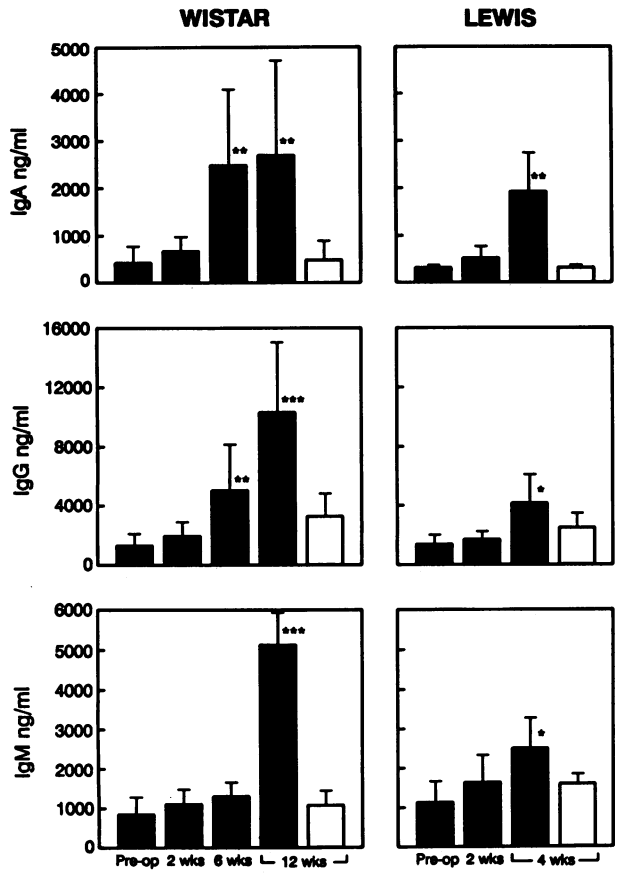

FIG. 2. Plasma anti-PG antibody levels in Lewis and Wistar rats with and without blind loops. All values are the mean ± standard deviation (SD) for at least eight rats per group. Plasma antibody levels were determined from tail vein blood prior to surgery and then at 2, 6, and 12 weeks for Wistar rats with blind loops and at 2 and 4 weeks for Lewis rats with blind loops. Pre-op, Antibody levels obtained prior to surgery. These values represent pooled data from sham-operated rats and those with SFBL. Symbols: ■, rats with SFBL; □, sham-operated rats at the end of the experiment; *, **, and ***, $P < 0.05$, 0.005, and 0.001, respectively, compared with preoperation levels.

TABLE 4. Enteral IgA in rats with and without blind loops

Specimen source and experimental group[a]	n	Total IgA (μg)[b]	Specific IgA (ng)[c]	% Specific IgA[d]
Jejunum				
Lewis				
SEBL/SHAM	7	106 ± 54[e]	8 ± 3[e]	7.5
SFBL	8	4,800 ± 2,080	378 ± 153	7.9
SFBL Gent	6	4,652 ± 1,890	427 ± 125	9.2
SFBL Poly	6	4,320 ± 899	428 ± 103	9.9
SFBL Met	7	4,917 ± 1,121	112 ± 92[e]	2.3
Wistar				
SEBL/SHAM	7	114 ± 79[e]	5 ± 4[e]	4.4
SFBL	5	7,740 ± 2,290[f]	1,172 ± 878	15.1
SFBL Met	5	6,594 ± 1,875	183 ± 163[g]	2.8
Cecum				
Lewis				
SEBL/SHAM	5	3,030 ± 1,880	112 ± 59[h]	3.7
SFBL	10	3,530 ± 1,020	231 ± 71	6.5
SFBL Met	5	3,240 ± 865	125 ± 49[h]	3.9
Wistar				
SEBL/SHAM	7	3,550 ± 1,310	116 ± 72[h]	3.3
SFBL	6	3,310 ± 1,420	258 ± 122	7.8
SFBL Met	5	3,122 ± 1,115	110 ± 86[h]	3.5

[a] Lewis rats and Wistar rats were tested 4 and 12 weeks, respectively, after surgery. SEBL/SHAM, Pooled data for controls. For other abbreviations, see Table 1, footnote *a*.

[b] Total IgA per segment of bowel, which means the entire contents of the loop of SEBL and SFBL rats and the volume flushed through 10 cm of proximal jejunum for sham-operated rats. All values are mean ± SD.

[c] All values are mean ± SD.

[d] (Specific IgA/total IgA) × 100.

[e] $P < 0.001$ compared with SFBL rats which received no antibiotic treatment.

[f] $P < 0.05$ for Wistar rats compared with Lewis rats.

[g] $P < 0.005$ compared with SFBL rats which received no antibiotic treatment.

[h] $P < 0.05$ compared with cecal IgA from SFBL rats which received no antibiotic treatment.

DISCUSSION

This study demonstrates that PG-APS injected into SFBL rats is absorbed more than that injected into sham-operated rats or rats which had blind loops and which were treated with metronidazole. Therefore, SBBO is associated with increased PG-APS absorption. The data derived from the area under the curve (Fig. 1) showed that rats which had SFBL and which were treated with metronidazole had PG-APS absorption similar to that of sham-operated rats and that both had approximately eightfold less absorption than that which occurred in SFBL rats with no antibiotic treatment. PG-APS is absorbed from the intestine and probably delivered to the liver via the portal vein, since the PG-APS level was higher in portal-venous blood than in tail vein blood in every rat examined.

As well, SBBO is associated with an elevated specific antibody response to endogenous bacterial cell-wall polymer PG in Lewis and Wistar rats, and metronidazole and tetracycline prevent this response. The antibody response was not due to infection in the blood, peritoneum, liver, or spleen, and the response did not correlate with the translocation of viable organisms to MLNs. This antibody response demonstrates the immunogenic potential of luminal normal flora bacterial cell wall polymers. The origin of specific anti-PG antibody cannot be determined from these data, but the antibody may arise from the gut-associated lymphoid tissue or from systemic sources, such as the spleen or marrow. The rise in anti-PG antibody required 6 to 12 weeks for Wistar rats, which is long after disaccharidase deficiency and protein-losing enteropathy have been established, perhaps indicating that the accumulation of the antigen as well as its absorption is important in induction of an antibody response.

Anti-lipid A, anti-soy protein, and anti-whole chow antibodies were only minimally and inconsistently increased in rats with SFBL, indicating that anti-PG antibody is not simply part of a generally increased response to luminal antigens accompanying SBBO and/or mucosal injury. The fact that anti-lipid A antibody levels were not elevated in rats with SFBL could indicate that lipopolysaccharide (LPS) is a weak immunogen compared with PG, that there is not much LPS absorbed in rats with SBBO, or that endogenous LPS in control rats has already stimulated a maximal immune response. Previously, we showed that there were increased specific luminal IgA antibodies to sonicates of *Escherichia*

coli, *Bacteroides vulgatus*, and mixed anaerobic flora from blind loops, but the antigenic sites on these sonicates were unknown (15).

Plasma anti-PG IgG and IgM increased naturally over time in sham-operated rats, since postoperation levels were significantly greater than preoperation levels. The development of natural antibodies to this ubiquitous bacterial antigen with increased lengths of exposure to intestinal PG-PS and maturation of the immune system suggests continuous, low-grade absorption of luminal PG-PS. Antibody responses to other bacterial antigens increase with age (2, 17). We have previously demonstrated that serum anti-PG antibody levels increased following feeding of PG-APS to mature rats (21) and that colonic PG-APS absorption is enhanced by acetic acid-induced mucosal injury (22). Serum anti-PG antibody levels are increased in Crohn's disease; are especially associated with extraintestinal manifestations, juvenile rheumatoid arthritis, and ankylosing spondylitis; and are possibly increased in patients who have undergone a jejunoileal bypass for morbid obesity who develop arthritis (7). These data support the hypothesis that absorption of toxic PG-PS from enteric bacteria occurs normally in small amounts but that it may have pathogenic significance in several idiopathic, chronic inflammatory conditions (20).

Since IgA levels in blind loops were approximately 30-fold higher than levels in SEBL and sham-operated jejunum segments, measurement of cecal IgA was a useful parameter because the ceca of the rats in the three groups were approximately the same size and contained similar amounts of total IgA. Cecal IgA may represent the passive accumulation of antibody from proximal mucosa and bile, but increased ratios of anti-PG IgA to total IgA in the ceca of rats with experimental SBBO may also indicate that the entire intestine has been sensitized to PG by jejunal bacterial overgrowth. Interestingly, treatment with metronidazole prevented increased anti-PG IgA concentrations within both the SFBL and the ceca.

Although SBBO may induce a polyclonal IgA response, an alternate explanation for elevated levels of total IgA in plasma in Lewis and Wistar rats is the hepatic injury induced by SBBO (13), since hepatic injury has been shown to result in higher plasma IgA levels in humans (11) and rats (12) because of decreased biliary secretion of secretory IgA and IgA-antigen complexes. However, the increase in anti-PG IgA was 450%, compared with only a 61% increase in total IgA in Wistar rats and a 17% increase in total IgA in Lewis rats. The anti-PG IgA level was also higher than the specific antibody response to lipid A and the dietary soy and chow antigens, thereby indicating an increased specific IgA immune response against PG rather than a nonspecific defect in biliary excretion of secretory IgA or a nonspecific polyclonal IgA response.

The ELISA for measuring PG-APS is very specific for the polysaccharide of group A streptococci and has minimal cross-reactivity with cell wall polymers derived from other bacteria. This fact was reported by Eisenberg et al. (6) with regard to the radioimmunoassay for PG-APS, which was the forerunner to our ELISA and utilizes the same antibody. We confirmed this by measuring PG-APS in plasma and liver homogenates from SFBL Lewis rats which were not injected with PG-APS and found these levels to be approximately 10 times lower than the lowest values obtained following PG-APS injection. The anti-PG assay, however, is not specific and should identify antibodies to PG from many bacterial sources, since two of three documented epitopes are found in almost all bacterial species. Streptococci were used as a source of PG that will cross-react with most bacterial species. We do not suggest that anti-PG antibodies arise from enteric streptococci but instead that anti-PG antibody reflects a response to luminal PG from a wide variety of bacterial species.

The elevated levels of plasma antibody are an indirect measure of increased absorption of PG. The increased ratio of anti-PG IgA to total IgA in plasma (450%) compared with the luminal ratio (200%) indicates that there is at least an element of systemic anti-PG antibody production. These data, in conjunction with direct measurement of transport of intraluminally injected PG-APS from the SFBL, support our hypothesis (13) that bacterial cell wall PG polymers derived from normal flora bacteria are absorbed from the intestinal tract in small amounts in the normal state, with enhanced transport under conditions which perturb gut permeability or increase luminal concentrations of PG-PS. Metronidazole and tetracycline decreased total luminal bacterial numbers only minimally but prevented disaccharidase deficiency. Recently, we showed that metronidazole and tetracycline treatment eliminated certain bacterial species which may have been responsible for mucosal injury (14). This confirms the data of Welkos et al. (31), who showed that metronidazole caused small changes in the numbers of anaerobic bacteria within blind loops but eliminated *Bacteroides* spp. Therefore, metronidazole and tetracycline may have prevented the development of elevated anti-PG antibody levels by decreasing intestinal injury typically induced by SBBO. Gentamicin and polymyxin B did not reverse mucosal injury (as demonstrated by low disaccharidase levels), did not eliminate *Bacteroides* spp. (14), and did not prevent elevated anti-PG antibody levels.

The creation of blind jejunal loops and the consequent large overgrowth of anaerobic bacteria both increases the luminal PG-PS concentration and induces mucosal injury. Enhanced absorption of these toxic endogenous bacterial cell wall polymers from the lumen may lead to hepatobiliary injury associated with intestinal bacterial overgrowth in susceptible rat strains (13) and may have implications for the pathogenesis of extraintestinal manifestations of human inflammatory bowel disease and jejunoileal bypass.

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REFERENCES

1. **Berg, R. D.** 1981. Promotion of the translocation of enteric bacteria from the gastrointestinal tracts of mice by oral treatment with penicillin, clindamycin, or metronidazole. Infect. Immun. **33**:854–861.
2. **Burgio, G. R., A. Lanzavecchia, A. Plebani, S. Jayakar, and A. G. Ugazio.** 1980. Ontogeny of secretory immunity: levels of secretory IgA and natural antibodies in saliva. Pediatr. Res. **14**:1111–1114.
3. **Cameron, D. G., G. M. Watson, and L. J. Witts.** 1949. The experimental production of macrocytic anemia by operations of the intestinal tract. Blood **4**:803–815.
4. **Cromartie, W. J., J. G. Craddock, J. H. Schwab, S. K. Anderle, and C. H. Yang.** 1977. Arthritis in rats after systemic injection of streptococcal cells or cell walls. J. Exp. Med. **146**:1585–1602.
5. **Dalqvist, A.** 1964. Methods for assay of intestinal disaccharidases. Anal. Biochem. **7**:18–25.
6. **Eisenberg, R., A. Fox, J. J. Greenblatt, S. K. Anderle, W. J. Cromartie, and J. H. Schwab.** 1982. Measurement of bacterial

cell wall in tissues by solid-phase radioimmunoassay: correlation of distribution and persistence with experimental arthritis in rats. Infect. Immun. **38**:127–135.
7. **Ely, P. H.** 1980. The bowel bypass syndrome: a response to bacterial peptidoglycans. J. Am. Acad. Dermatol. **2**:473–493.
8. **Gut, J. P., S. Schmitt, A. Bingen, B. Anton, and A. Kirn.** 1984. Probable role of endogenous endotoxins in hepatocytolysis during murine hepatitis caused by frog virus 3. J. Infect. Dis. **149**:621–629.
9. **King, C. E., and P. P. Toskes.** 1979. Small intestine bacterial overgrowth. Gastroenterology **76**:1035–1055.
10. **King, C. E., and P. P. Toskes.** 1981. Protein-losing enteropathy in the human and experimental rat blind-loop syndrome. Gastroenterology **80**:504–509.
11. **Kutteh, W. H., S. J. Prince, J. O. Phillips, J. G. Spenney, and J. Mestecky.** 1982. Properties of immunoglobulin A in serum of individuals with liver diseases and in hepatic bile. Gastroenterology **82**:184–193.
12. **Lemaitre-Coelho, I., G. D. F. Jackson, and V. P. Vaerman.** 1978. Relevance of biliary IgA antibodies in rat intestinal immunity. Scand. J. Immunol. **8**:459–463.
12a. **Lichtman, S. N., J. Keku, R. B. Sartor, R. Clark, and J. H. Schwab.** Hepatology, in press.
13. **Lichtman, S. N., J. Keku, R. B. Sartor, and J. H. Schwab.** 1989. Hepatic injury associated with small bowel bacterial overgrowth in rats. Gastroenterology **98**:414–423.
14. **Lichtman, S. N., J. Keku, J. H. Schwab, and R. B. Sartor.** Gastroenterology, in press.
15. **Lichtman, S. N., P. Sherman, and G. Forstner.** 1986. Production of secretory IgA in rat self filling blind loops: local sIgA immune response to luminal bacterial flora. Gastroenterology **91**:1495–1502.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Mellander, L., B. Carlsson, and L. A. Hanson.** 1984. The appearance of secretory IgM and IgA antibodies to E. coli in saliva during infancy and childhood. J. Pediatr. **104**:564–568.
18. **Menge, H., R. Kohn, K. H. Dietermann, H. Lorenz-Meyer, E. D. Reichen, and J. W. L. Robinson.** 1979. Structural and functional alterations in the mucosa of self-filling blind-loops in rats. Clin. Sci. **56**:121–131.
19. **Nolan, J. P., and A. I. Leibowitz.** 1978. Modification of acute carbon tetrachloride injury by polymyxin B—an antiendotoxin. Gastroenterology **75**:445–449.
20. **Sartor, R. B.** 1989. Importance of intestinal mucosal immunity and luminal bacterial cell wall polymers in the etiology of inflammatory joint diseases. Bailliere's Clin. Rheum. **3**:223–245.
21. **Sartor, R. B., T. M. Bond, K. Y. Compton, and D. R. Cleland.** 1987. Intestinal absorption of bacterial cell wall polymers in rats. Adv. Exp. Med. Biol. **216A**:835–839.
22. **Sartor, R. B., T. M. Bond, and J. H. Schwab.** 1988. Systemic uptake and intestinal inflammatory effects of luminal bacterial cell wall polymers in rats with acute colonic injury. Infect. Immun. **56**:2101–2108.
23. **Sartor, R. B., W. J. Cromartie, D. W. Powell, and J. H. Schwab.** 1985. Granulomatous enterocolitis induced in rats by purified bacterial cell wall fragments. Gastroenterology **89**:587–595.
24. **Severijnen, A. J., M. P. Hazenberg, and J. P. van de Merwe.** 1988. Induction of chronic arthritis in rats by cell wall fragments of anaerobic coccoid rods isolated from the fecal flora of patients with Crohn's disease. Digestion **39**:118–125.
25. **Sherman, P., and S. Lichtman.** 1987. Small bowel bacterial overgrowth syndrome. Surv. Dig. Dis. **5**:157–171.
26. **Sherman, P., A. Wesley, and G. Forstner.** 1985. The sequential disaccharidase loss in rat intestinal blind loops: impact of malnutrition. Am. J. Physiol. **248**:G626–G632.
27. **Stimpson, S. A., R. R. Brown, S. K. Anderle, D. G. Klapper, R. L. Clark, W. J. Cromartie, and J. H. Schwab.** 1986. Arthropathic properties of cell wall polymers from normal flora bacteria. Infect. Immun. **51**:240–249.
28. **Stimpson, S. A., J. H. Schwab, M. J. Janusz, S. K. Anderle, R. R. Brown, and W. J. Cromartie.** 1986. Acute and chronic inflammation induced by peptidoglycan structures and polysaccharide complexes, p. 273–290. *In* P. H. Seidl and K. H. Schleifer (ed.), Biological properties of peptidoglycan. Walter de Gruyter & Co., Berlin.
29. **Toskes, P. P., R. A. Gianella, H. R. Jervis, W. R. Rout, and A. Takeuchi.** 1975. Small intestinal mucosal injury in the experimental blind-loop syndrome: light and electron microscopic and histochemical studies. Gastroenterology **68**:1193–1203.
30. **Wahl, S. M., D. A. Hunt, J. B. Allen, R. L. Wilder, L. Paglia, and A. R. Hand.** 1986. Bacterial cell wall induced hepatic granulomas. J. Exp. Med. **163**:884–902.
31. **Welkos, S. L., P. P. Toskes, H. Baer, and G. W. Smith.** 1981. Importance of anaerobic bacteria in the cobalamin malabsorption in the experimental rat blind loop syndrome. Gastroenterology **80**:313–320.