Protection against Experimental Intraabdominal Sepsis by Two Polysaccharide Immunomodulators

Arthur O. Tzianabos, Frank C. Gibson III, Ronald L. Cisneros, and Dennis L. Kasper

Channing Laboratory, Brigham and Women's Hospital, Departments of Medicine and Pathology, Harvard Medical School, Boston, Massachusetts

Two immunomodulating polysaccharides, poly-(1-6)-β-glucotriosyl-(1-3)-β-glucopyranose (PGG)-glucan and *Bacteroides fragilis* polysaccharide A (PS A), were evaluated for the prevention of mortality and abscess formation associated with experimental intraabdominal sepsis. Prophylactic treatment with a combination of these compounds significantly reduced mortality (8% vs. 44% in the saline-treated control group) and the incidence of abscesses (30% vs. 100% in the saline-treated control group) after challenge with rat cecal contents. These compounds were also effective when administered therapeutically after bacterial contamination of the peritoneal cavity. PS A treatment conferred long-term protection against abscess formation and resulted in significantly fewer total aerobes and anaerobes in the peritoneal fluid of animals challenged with cecal contents. These data demonstrate the usefulness of two immunomodulatory polysaccharides in preventing experimental intraabdominal sepsis in the absence of antimicrobial therapy and may represent a new adjunct to antibiotic regimens currently used to prevent clinical cases of this disease.

Bacterial contamination of the abdominal cavity usually occurs after perforation of the large bowel due to penetrating trauma to the abdomen, as a complication subsequent to abdominal surgery, or as a result of underlying bowel disease. Numerous clinical and experimental studies have shown that the release of colonic contents into the peritoneal cavity can lead to widespread septicemia and/or the formation of intraabdominal abscess(es) [1–3]. The advent of prophylactic or therapeutic treatment with antimicrobial agents with activity against both the facultative and obligate anaerobic components of the intestinal flora has greatly reduced the infection rate associated with intraabdominal sepsis. However, despite appropriate treatment with antibiotics, a significant number of abdominal infections still occur in the clinical setting. Abdominal surgery and postoperative wound infection rates range from 10% to 20%, while the incidence of abscesses in patients is 1%–4% [1]. The failure of antimicrobial therapy in these cases may be attributable to many factors, including the emergence of antibiotic-resistant bacteria and poor penetration of these agents within the abdominal cavity.

Previous studies in a rat model of intraabdominal sepsis have documented the role of particular intestinal bacterial species that predominate in experimental intraabdominal sepsis [3–6]. Facultative species, such as *Escherichia coli,* predominate in the septic phase of disease and are associated primarily with mortality, while anaerobes, such as *Bacteroides fragilis,* are associated with abscess formation. In addition, a synergistic relationship between facultative and obligate anaerobes was defined that suggested that sepsis and abscess formation associated with this disease process was mediated, at least in part, by the interaction of these bacterial species [6–10].

Previous studies have established the efficacy of soluble polymers of poly-(1-6)-β-glucotriosyl-(1-3)-β-glucopyranose (PGG)-glucan (Betafectin; Alpha-Beta Technology, Worcester, MA), a biologic response modifier, in prophylactic protection against bacteremia and mortality associated with experimentally induced peritonitis in rodent models [7, 9]. In these studies, prophylactic treatment with PGG-glucan yielded enhanced clearance of bacteria from the peritoneal cavities of rats and increased total leukocyte counts. Protection against sepsis in this model was mediated, at least in part, by the induction of prostaglandin production [9].

Work with the capsular polysaccharide complex of *B. fragilis* has demonstrated the importance of this virulence factor in regulating intraabdominal abscess formation in the rat model [11–14]. Further studies have shown that the positively and negatively charged groups associated with the two component polysaccharides of the capsular polysaccharide complex, polysaccharide A (PS A) and polysaccharide B (PS B), mediate their ability to both induce abscess formation and protect against this host response in the animal model. Structurally distinct polysaccharides that possess this dual charge motif, such as the *Streptococcus pneumoniae* type 1 capsule, exhibit the same biologic properties as PS A and PS B, while other polysaccharides that are uncharged or have only negatively charged groups do not [12–14]. Recently, we have shown that short-term pro-

phylactic administration of the most active of these polysaccharides, PS A, reduces the incidence of abscesses in rats challenged with ''abscessogenic'' synergistic combinations of facultative and obligate anaerobes or with a cecal contents inoculum [11]. Further, this protective activity was mediated by T cells. These studies suggested that PS A possessed immunomodulatory activities distinct from an antigen-specific anamnestic host response.

Previous studies suggest that prophylactic PGG-glucan and PS A treatment could be useful in preventing both mortality and abscess formation associated with experimental intraabdominal sepsis. However, it is clear that treatment with these immunomodulating polysaccharides would be more beneficial if used in the therapeutic mode, since the greatest risk of intraabdominal sepsis is associated with clinical cases of unexpected bowel perforation, such as in patients with underlying bowel disease, diverticulitis, appendicitis, and penetrating abdominal trauma [15]. To date, no compounds other than antibiotics have been used in experimental models to successfully prevent both phases of this disease process, and little is known regarding the therapeutic usefulness of these immunomodulators.

In the present study, we investigated the use of PGG-glucan and PS A alone and in combination to prevent experimental intraabdominal sepsis. The compounds were evaluated for their ability to reduce the incidence of both mortality and abscess formation associated with disease when used in either the prophylactic or therapeutic mode. Finally, studies were done to better delineate the protective activity of the newly described immunomodulator, PS A.

Materials and Methods

Animal model for intraabdominal sepsis and challenge inocula. An animal model for intraabdominal sepsis was used for these studies [5]. Briefly, male Wistar rats (180–200 g; Charles River Laboratories, Wilmington, MA) were anesthetized with a single intraperitoneal injection of 0.15 mL of Nembutal (50 mg/mL; Abbott Laboratories, North Chicago, IL). An anterior midline incision (0.5 cm) was made through the abdominal wall and peritoneum, and a gelatin capsule with 0.5 mL of inoculum was inserted into the pelvis. Two types of inocula were used for these experiments. In studies to assess protection against *B. fragilis*–induced abscesses, an axenic culture of *B. fragilis* NCTC 9343 (10^8 cfu/rat) was mixed with sterile rat cecal contents and 10% (wt/vol) barium sulfate. For studies to produce mortality and abscess formation associated with intraabdominal sepsis, a rat cecal contents inoculum was used as previously described [3, 11]. The cecal contents inoculum was procured from the ceca of meat-fed rats, mixed with Luria broth to obtain a slurry, and frozen at $-80°C$ until needed. Quantitative and qualitative bacteriology of this inoculum was done as previously described [11]. For animal experiments, the cecal contents inoculum was mixed with barium sulfate (10% final concentration [wt/vol]) and titrated in the rat model to yield ~50% mortality and 100% abscess formation in survivors. Animals were examined daily and mortality rates assessed in each treatment

group. Animals typically succumbed to the acute phase of sepsis within 48 h after challenge, while abscess formation required 6 days. Six days after challenge, surviving animals were necropsied in an observer-blinded fashion and examined for intraabdominal abscesses. The presence of one or more abscesses in an animal was scored as a positive result. Results are reported as a compilation of at least two separate experiments.

Polysaccharides and treatment regimens. PGG-glucan, a soluble polymer of poly-(1-6)-β-glucotriosyl-(1-3)-β-glucopyranose, purified from genetically engineered yeast cells, was prepared in sterile, pyrogen-free saline solution. For prophylactic administration, the solution was administered by intramuscular injection 48, 24, and 4 h before challenge and 4 h after surgical implantation of the bacterial inoculum. A dosage of 100 μg/animal/injection administered in sterile pyrogen-free saline via the intramuscular route was used for all experiments. For therapeutic administration, PGG-glucan was administered 4 and 24 h after challenge.

B. fragilis PS A was prepared as previously described [11, 16, 17]. PS A was prepared in sterile, pyrogen-free saline, and designated doses were administered to animals by the subcutaneous route according to treatment regimens (see Results). In all experiments, a group of animals was treated with the saline vehicle used for polysaccharide treatment and served as the negative control.

Quantitative peritoneal fluid cultures. Peritoneal fluid was obtained from saline- and PS A–treated animals at designated time intervals and diluted in sterile PBS to yield decimal dilutions ranging from 10^{-2} to 10^{-7}, and aliquots were plated onto Brucella-based or T soy blood plates (Difco, Detroit). Plates were placed in an aerobic or anaerobic environment to assess total aerobic and anaerobic bacterial counts. *B. fragilis* was classified by gas-liquid chromatographic analysis of glucose fermentation products and antibiotic susceptibility patterns. Final identification included the use of PRAS II biochemical tests (Randolph Biomedical, West Warwick, RI) and analysis of long chain fatty acids by use of the Microbial Identification System (MIDI, Newark, DE).

Results

Prophylactic combination treatment with PGG-glucan and PS A. The effect of prophylactic combination treatment with PGG-glucan and PS A in reducing the incidence of mortality associated with intraabdominal sepsis was first examined. Animals were treated with saline, PGG-glucan, PS A, or a combination of the two compounds before and after challenge with the cecal contents inoculum as designated in figure 1. Treatment with PGG-glucan alone resulted in a significant reduction in mortality compared with the saline-treated control group (17% vs. 44%; $P < .05$; figure 1A). Treatment with PS A alone reduced mortality from 44% to 32%, but this reduction was not statistically significant. Treatment with the combination of PGG-glucan and PS A yielded the lowest mortality rate (8%) of all the treated groups ($P < .002$ compared with the saline-treated control group; $P = .051$ compared with the PS A–treated group).

Surviving animals from each of the treatment groups were then examined for the presence of intraabdominal abscesses

A <u>Treatment</u>

B <u>Treatment</u>
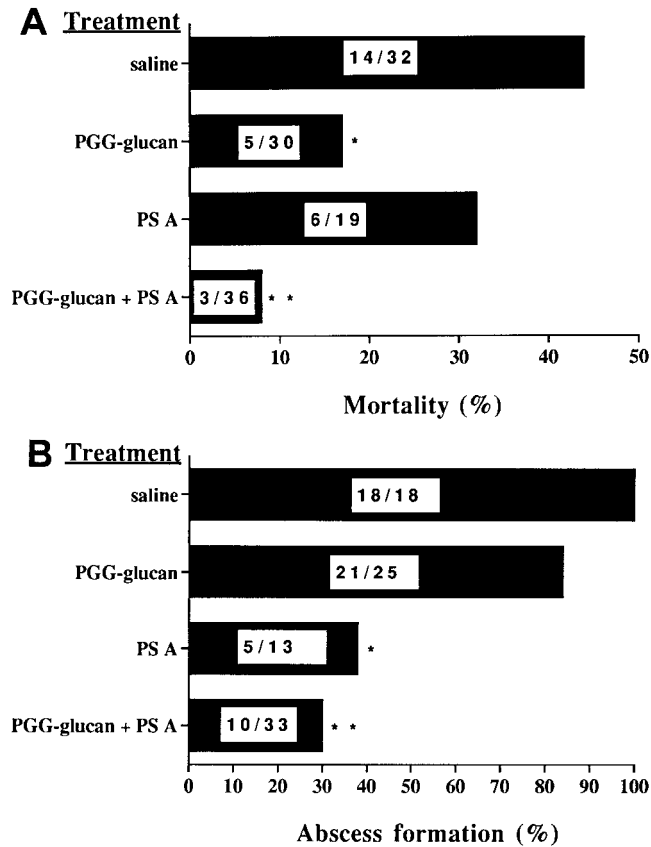
Figure 1. Polysaccharide-mediated protection against mortality and abscess formation induced by cecal contents challenge. Animals were treated prophylactically with PGG-glucan (-48, -24, -4, and $+4$ h relative to challenge) and/or PS A (-24, $+1$, $+24$, and $+72$ h relative to challenge) and challenged with rat cecal contents inoculum. **A**, Mortality rates in groups of animals treated with PGG-glucan, PS A, or combination. Combination treatment induced highest level of protection. * $P < .05$; ** $P < .002$ vs. saline-treated control, Fisher's exact test. **B**, Abscess formation rates in groups of animals treated with PGG-glucan, PS A, or combination. Treatment with both PGG-glucan and PS A induced highest level of protection. * $P < .001$; ** $P < .001$ vs. saline-treated control, Fisher's exact test.

(figure 1B). Treatment with PS A alone resulted in a significant reduction in abscess formation compared with the saline-treated control group (38% vs. 100%; $P = .001$), while treatment with PGG-glucan alone lowered the abscess rate to 84%, a reduction that was not statistically significant. Treatment with the combination of PGG-glucan and PS A was the most effective regimen, reducing the incidence of abscesses to 30% ($P < .001$ compared with the saline-treated control group; $P < .001$ compared with the PGG-glucan–treated group).

Therapeutic (postchallenge) treatment with PGG-glucan or PS A.   The efficacy of therapeutic treatment of rats with PGG-glucan for the prevention of mortality was evaluated. Animals were treated with 100 μg of PGG-glucan 4 and 24 h after challenge with the cecal contents inoculum. Animals treated with PGG-glucan had a lower mortality rate than did the saline-

treated control group (30% vs. 60%; $P < .005$). Delay of PGG-glucan treatment >4 h after challenge failed to protect animals against mortality associated with cecal contents challenge. In addition, increasing the concentration of PGG-glucan from 100 μg to 400 μg/dose failed to enhance the protection against mortality in these experiments (data not shown).

To determine whether PS A therapy could protect against abscess formation induced by the cecal contents inoculum, animals were challenged and treated with 50 μg of PS A 1, 24, 48, and 96 h after surgery. Results from these experiments showed that significantly fewer animals developed abscesses in the PS A–treated group than in the saline group (25% vs. 84%; $P < .001$).

Delayed therapeutic protection against abscess formation by PS A.   The chronic phase of intraabdominal sepsis, abscess formation, requires a period of ≥ 5 days after challenge to develop in the rat model. This period of time allowed for the opportunity to intervene with multiple doses of PS A after challenge and before the development of abscesses. In initial experiments, animals were challenged with *B. fragilis* to produce only the abscess phase of disease and given three different PS A regimens (designated in figure 2). The first regimen delayed the onset of therapeutic treatment by 1 h after challenge, while the second and third regimens delayed the onset of PS A treatments by 24 h or 48 h, respectively. While delaying PS A treatment for 1 h or 24 h after challenge with *B. fragilis* still induced significant protection against abscess formation, delaying treatment for 48 h before initiation of PS A therapy did not protect animals against abscess formation (figure 2). Animals given PS A treatment 1 or 24 h after challenge had significantly lower abscess rates of 21% and 40%, respectively,

<u>Treatment</u>
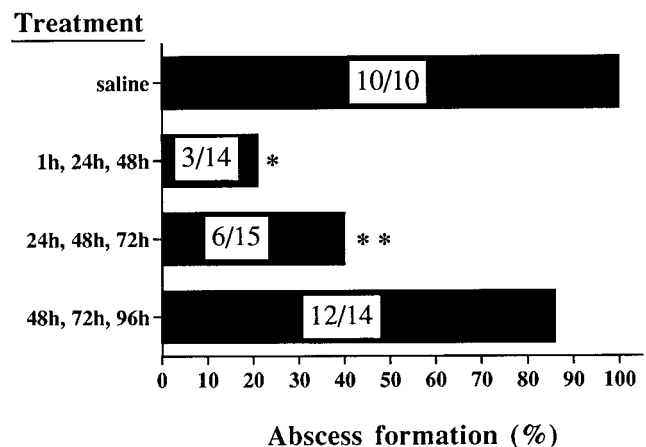
Figure 2. Delayed therapeutic protection against abscess formation by PS A. Animals were challenged with *B. fragilis* (10^8 cfu/mL) and treated with 3 doses of PS A (10 μg/dose) or saline at designated time intervals. Animals were sacrificed 6 days later and examined for presence of intraabdominal abscesses. PS A treatment could be delayed for as long as 24 h after challenge and still induce protection against abscess formation. Delay of treatment to 48 h failed to elicit significant protection. * $P < .001$; ** $P < .005$, Fisher's exact test.
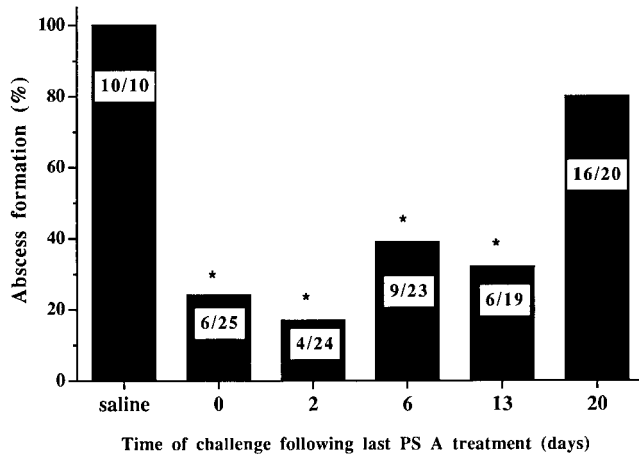
Figure 3. Duration of protective activity by PS A. Groups of animals were given 3 doses of PS A (10 μg/dose) on 3 successive days and challenged with *B. fragilis* (10^8 cfu/mL) at day 0 (concurrent with final PS A dose), 2, 6, 13, or 20 after last dose administered. Holding animals for this additional length of time does not affect their ability to form abscesses with dose of *B. fragilis* used. Animals were then examined for abscess formation 6 days after challenge. Animals challenged at day 0, 2, 6, and 13 had significantly fewer abscesses than saline-treated controls, while animals challenged 20 days later were not protected. * $P < .005$, Fisher's exact test.

compared with 100% in the saline-treated control group. The delay of PS A treatment for 48 h after challenge yielded an abscess rate of 86%.

Duration of protective activity by PS A. To determine the duration of the protective activity of PS A, animals were given a three-dose regimen of the polysaccharide (10 μg/dose) and challenged at various times after the final dose of polysaccharide (0, 2, 6, 13, or 20 days after treatment). We have previously established that holding animals for this additional length of time does not affect their ability to form abscesses with the dose of *B. fragilis* used. Results are shown in figure 3. All saline-treated animals formed one or more intraabdominal abscesses, while animals treated with the last dose of PS A at the time of challenge had a 24% abscess rate ($P < .001$). Groups of animals challenged 2, 6, and as long as 13 days after the final PS A treatment had significantly lower abscess rates compared with the saline-treated control group ($P < .005$). In contrast, animals challenged 20 days after the last dose of PS A were not protected against abscess formation (80% abscess rate).

Bacterial clearance from the peritoneal cavity of PS A–treated animals. To investigate whether the prevention of abscesses by PS A in this model could be related to the enhancement of bacterial clearance from the peritoneal cavities of rats, bacterial counts were done on peritoneal fluid cultures of animals challenged with monomicrobial or polymicrobial inocula. Initially, animals were prophylactically treated with PS A (10 μg/dose) at -24, $+1$, $+24$, $+48$, and $+72$ h relative to challenge with *B. fragilis* (10^8 cfu/animal). Peritoneal fluid

cultures were taken from PS A$-$ and saline-treated animals at 24, 72, 96, and 120 h after challenge, and the total count of organisms was determined. *B. fragilis* persisted within the peritoneal cavities of saline-treated animals at $\sim 10^7$ cfu/mL until 120 h after challenge, when bacterial counts declined to $10^{5.97}$ cfu/mL (figure 4A). In contrast, there was enhanced bacterial clearance in the PS A–treated rats: Bacterial counts were lower than in saline-treated animals at 72 and 96 h after challenge and significantly reduced to $10^{3.92}$ cfu/mL 120 h after challenge (figure 4A).

To extend these findings, we did similar experiments to determine the effect of PS A treatment on the clearance of aerobic and anaerobic bacteria from the peritoneal cavities of rats after challenge with the cecal contents inoculum. Animals were treated prophylactically with PS A (50 μg/dose) and challenged with cecal contents inoculum, and peritoneal fluid cultures were taken from PS A$-$ and saline-treated animals 24, 72, 96, and 120 h after challenge. Total aerobic and anaerobic bacterial counts in both saline- and PS A–treated animals declined within the first 24 h after challenge, were increased at 72 and 96 h after challenge, and declined again at 120 h after challenge (figures 4B, C). Although there were no significant differences in total number of aerobes or anaerobes between saline- and PS A–treated animals at the 24, 72, or 96 h time points, there were significant differences in PS A–treated animals 120 h after challenge. The number of total aerobes in saline-treated animals at this time point was $10^{3.6}$ cfu/mL, compared with $10^{2.15}$ cfu/mL in PS A–treated animals (figure 4B; $P < .05$). The total number of anaerobes found in saline-treated animals at this time point was $10^{5.3}$ cfu/mL, while the number of anaerobes found in PS A–treated animals was below the limit of detection ($<10^2$ cfu/mL) for this assay (figure 4C).

Discussion

Because of the emergence of antibiotic-resistant bacteria and the continued incidence of postsurgical infections associated with high-risk abdominal procedures, investigators have begun to identify potential compounds that could augment standard antibiotic regimens currently used in these cases. In this study, we evaluated the use of two immunomodulatory polysaccharides for the prevention of experimental intraabdominal sepsis.

We first evaluated the prophylactic use of PGG-glucan and PS A alone and in combination for their ability to reduce the incidence of both mortality and abscess formation associated with experimental intraabdominal sepsis. This inoculum is prepared from meat-fed rats and closely approximates the microflora of human intestinal contents [3]. Intraperitoneal challenge with rat cecal contents simulates the release of human colonic contents into the abdominal cavity following rupture or leakage of the bowel. The results of these experiments clearly demonstrate that combination treatment with the two immunomodulating polysaccharides was the best regimen for the prevention of both
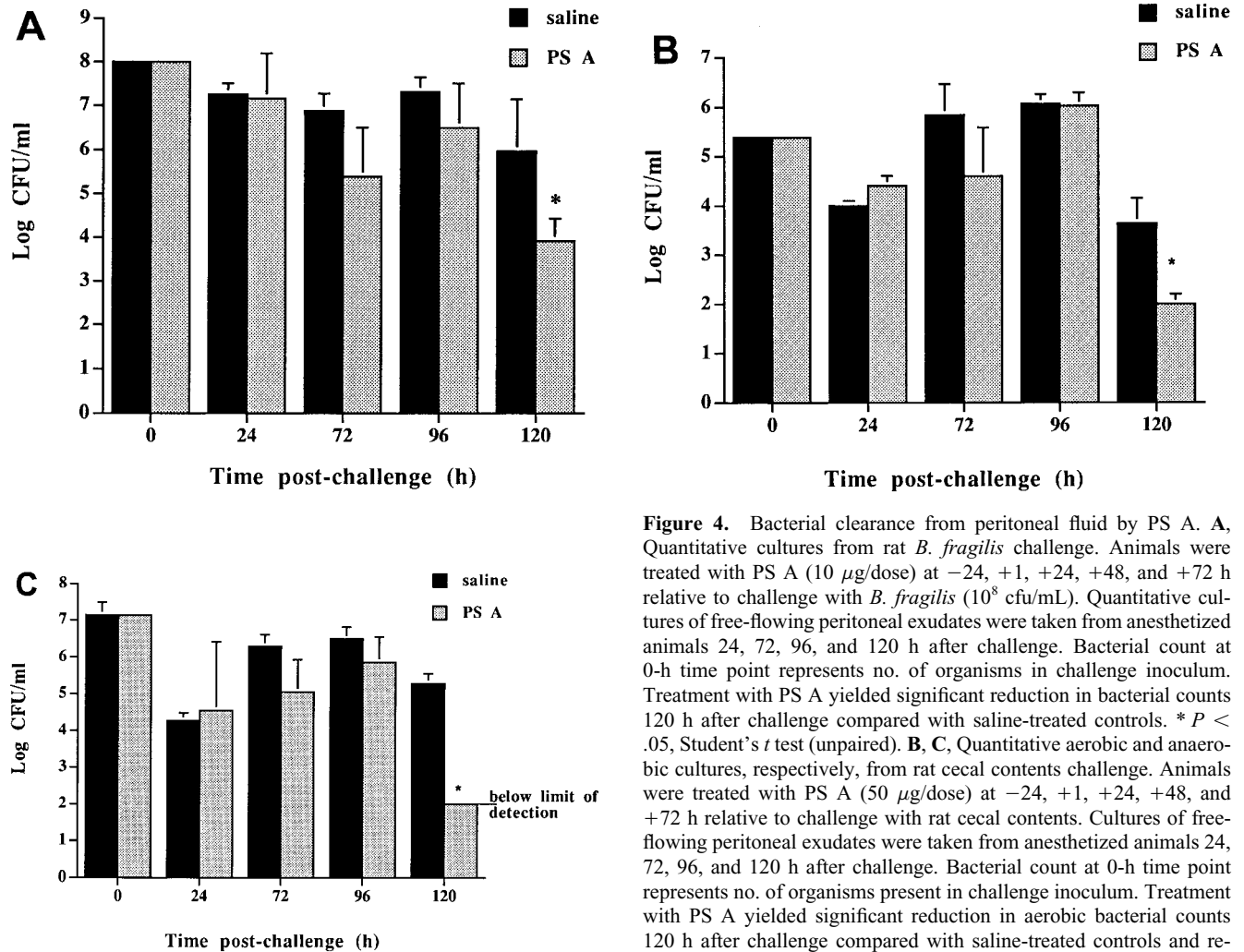
Figure 4. Bacterial clearance from peritoneal fluid by PS A. **A**, Quantitative cultures from rat *B. fragilis* challenge. Animals were treated with PS A (10 μg/dose) at -24, $+1$, $+24$, $+48$, and $+72$ h relative to challenge with *B. fragilis* (10^8 cfu/mL). Quantitative cultures of free-flowing peritoneal exudates were taken from anesthetized animals 24, 72, 96, and 120 h after challenge. Bacterial count at 0-h time point represents no. of organisms in challenge inoculum. Treatment with PS A yielded significant reduction in bacterial counts 120 h after challenge compared with saline-treated controls. * $P <$ .05, Student's *t* test (unpaired). **B**, **C**, Quantitative aerobic and anaerobic cultures, respectively, from rat cecal contents challenge. Animals were treated with PS A (50 μg/dose) at -24, $+1$, $+24$, $+48$, and $+72$ h relative to challenge with rat cecal contents. Cultures of free-flowing peritoneal exudates were taken from anesthetized animals 24, 72, 96, and 120 h after challenge. Bacterial count at 0-h time point represents no. of organisms present in challenge inoculum. Treatment with PS A yielded significant reduction in aerobic bacterial counts 120 h after challenge compared with saline-treated controls and reduced no. of anaerobic organisms in peritoneal fluid of treated animals to below detection limit for this assay (10^2 cfu/mL). * $P <$ .05, Student's *t* test (unpaired).

phases of experimental intraabdominal sepsis. These data demonstrated that PGG-glucan was most effective in preventing mortality, while PS A was most effective in reducing the incidence of abscess formation. It was interesting to note that PGG-glucan and PS A had some crossover activity in reducing abscess formation and mortality, respectively, but the level of reduction was not statistically significant in either case. Although a number of biologics, such as monoclonal antibodies, cytokines, and receptor antagonists, have been tested to reduce the incidence of sepsis in animal models and clinical trials, this is the first example of compounds other than antibiotics that can be successfully used to lower the incidence of both mortality and abscess formation in experimental intraabdominal sepsis.

It is clear that the highest risk of postoperative infections occurs in patients with unexpected bowel perforation, such as in cases of penetrating abdominal trauma, appendicitis, or diverticulitis or after development of underlying disease [15].

Therefore, we investigated the potential of using each of these compounds in the therapeutic mode to ascertain their effect on each phase of intraabdominal sepsis. Experiments with PGG-glucan showed that it could be administered a short time (4 h) after challenge with cecal contents and still induce a protective response in animals. These results were surprising, given the fact that the onset of sepsis in this model is rapid and severe, with most animals dying $18-24$ h after challenge. It is likely that PGG-glucan induces a rapid immunomodulatory effect that adequately dampens the septic phase of the disease process. Delay of the administration of PGG-glucan beyond this time failed to elicit protection, indicating that while a protective response most likely occurs within a short period of time, the onset of frank sepsis can overwhelm this protective effect in the absence of antibiotic therapy. Further studies are underway to assess the use of PGG-glucan treatment as an adjunct to antibiotic therapy in these cases.

Studies with PS A were then done to ascertain its usefulness when used in the therapeutic mode for the prevention of intraabdominal abscesses. Previous studies had indicated that PS A could be used in a therapeutic mode to protect against monomicrobial challenge with *B. fragilis* [11]. However, it was unclear whether this compound could be used in a setting that more closely approximated the clinical situation, in which peritoneal soilage is caused by the release of several hundred bacterial species into the abdomen. In these studies, PS A could be administered as late as 24 h after challenge with cecal contents and still protect against abscess formation. This protective activity was lost when administration was delayed to 48 h after challenge. These data supported our belief that therapeutic protection against abscess formation was possible in the animal model, as this pathologic host response takes at least 4–5 days to form. In contrast to the rapid onset of sepsis in this model, the prolonged formation of abscesses in rats provides ample time for the commencement of therapeutic intervention for the prevention of this host response. Given the fact that abscess formation in people also occurs as a chronic infection over an extended period of time, the window of opportunity for therapeutic intervention with PS A exists, and successful therapy could be possible.

We next examined the length of time PS A–mediated protection persisted in animals after the last dose of polysaccharide administered. The finding that this protective activity lasted for as long as 2 weeks after the final dose indicated that PS A may have long-lived biologic effects. This finding contrasts with previous results with PGG-glucan in rodent models. The protective activity of this polysaccharide is short-lived and depends on repeated administration to animals [7]. Whether the long-lived effects of PS A are due to limited clearance from the circulating lymph system or the induction of a lasting immune response is not known. We are currently investigating the pharmacokinetics of PS A distribution in animals.

Previous work with PGG-glucan has shown that the mechanism of protection is related in part to its ability to enhance bacterial clearance from treated animals after intraperitoneal challenge with rat cecal contents [7]. In these experiments, PGG-glucan treatment significantly reduced the number of bacteria in free-flowing peritoneal exudates from animals 96 h after challenge compared with that in saline-treated animals. In the present work, we tested whether the protective activity of PS A resulted in bacterial clearance in animals challenged with *B. fragilis* or the rat cecal contents inoculum. Animals treated prophylactically with PS A and challenged with *B. fragilis* had significantly lower bacterial counts in peritoneal fluid 120 h after challenge than did saline-treated animals. Further, animals receiving prophylaxis with PS A and challenged with the rat cecal contents inoculum had significantly fewer total aerobic and anaerobic organisms at this time point after challenge. In our animal model, this is

1–2 days before the time when fully formed abscesses are found in similarly challenged saline-treated or untreated animals. It is possible that PS A suppresses host factors that lead to the development of abscesses and results in improved mechanical clearance of the bacterial load via the diaphragmatic lymph system present in the peritoneal cavity.

A number of studies have documented the immunomodulatory properties of soluble glucans in protecting against experimentally induced peritonitis [7, 9, 18, 19]. These studies have focused mainly on the effects of prophylactic administration of these compounds on the acute phase of peritonitis but have not assessed the ability of this compound to prevent abscess formation associated with this disease process. Studies suggest that the protective effect of PGG-glucan is related to its ability to potentiate or prime the microbicidal activities of leukocytes [20]. In addition, protection against lethal challenge in the rat model has been shown to involve production of antiinflammatory mediators such as prostaglandins [9]. In contrast, *B. fragilis* PS A has only recently been described as an immunomodulating polysaccharide, and little is known about its protective activity. Previous studies in rats have shown that the protective activity conferred by PS A is mediated by T cells [11, 14]. We are currently investigating the interaction of PS A with this lymphocyte and the role of this interaction with abscess prevention.

In summary, these data suggest that the combined use of PGG-glucan and PS A could lower infection rates associated with abdominal surgeries. The use of immunomodulatory compounds that enhance the host's ability to combat infections represents a change from conventional antiinfective strategies that have relied solely on direct bactericidal activities of drug compounds. This approach would also be advantageous in cases involving antibiotic-resistant organisms and could be useful as an adjunct to standard antimicrobial therapy in intraabdominal sepsis as well as other infectious processes. Further work is being done to better understand the mechanisms by which these compounds interact with the host immune system to induce these protective effects.

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