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Arthropathic Properties of Cell Wall Polymers from Normal Flora Bacteria

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Peptidoglycan-polysaccharide (PG-PS) fragments were purified from cell walls of group D streptococci (*Streptococcus faecium*, strains ATCC 9790 and F-24) with a protocol which minimizes autolytic activity and tested for ability to induce arthritis in rats. PG-PS fragments from cell walls of other normal flora bacteria (*Peptostreptococcus productus*, and *Propionibacterium acnes*), group A streptococci, and pseudomurein-PS fragments from cell walls of *Methanobacterium formicicum*, were similarly purified and tested. Upon intraarticular injection into rat ankles, all PG-PS polymers induced acute inflammation; pseudomurein-PS fragments were approximately five times less active than the PG-PS preparations. After intraperitoneal injection, *P. acnes* PG-PS induced a minimal acute arthritis, *Peptostreptococcus productus* PG-PS induced a moderately severe acute joint inflammation followed by a mild chronic arthritis, and both group A and group D streptococcal PG-PS induced severe acute arthritis which evolved into chronic, erosive joint disease; pseudomurein-PS fragments were without effect, consistent with a crucial role for the PG moiety of PG-PS. Chronic arthritis induced by group D streptococcal PG-PS subsided after 60 days, whereas that induced by group A streptococcal PG-PS was still active after 128 days. The arthropathic properties of this modest number of common normal flora bacteria suggest that different PG-PS structures derived from the normal flora have the potential to induce a wide range of responses, from transient acute to chronic erosive joint disease.

A single intraperitoneal (i.p.) injection of a sterile, aqueous suspension of peptidoglycan-polysaccharide (PG-PS) fragments purified from group A streptococcal cell walls induces a chronic, erosive, recurrent polyarthritis in rats which resembles human rheumatoid arthritis in clinical, histologic, and radiologic detail (13). Based on the chronic arthropathic activity of sonically treated material of several other gram-positive bacteria (13) and the ubiquitous natural distribution of PG-containing polymers, Schwab (36) and Cromartie (12) hypothesized that cell wall polymers from many bacteria, including normal flora species, might possess arthropathic activity, and further postulated that increased exposure to these polymers during intestinal injury might be relevant to the association of chronic arthritis with ulcerative colitis, Crohn's disease, Whipple's disease, and intestinal bypass surgery for obesity (2). These ideas are supported by recent reports of chronic erosive arthritis induced in rats by cell wall polymers from group B streptococci (43) and *Lactobacillus casei* (29).

The present study was undertaken to extend our knowledge of the arthropathic properties of cell wall polymers from normal flora bacteria and to take advantage of the natural structural diversity of these polymers in the evaluation of structure-function relationships. Previous studies of the group A streptococcal PG-PS indicated that the relative severity of acute and chronic phases of arthritis was determined by PG-PS fragment size, which could be altered by physical (i.e., sonication) or enzymatic (i.e., muralytic digestion) means (8, 20). Therefore, cell wall polymers were purified by a protocol which minimizes autolytic activity. In addition, fragments were prepared which were similar in size to those of group A streptococcal PG-PS which induce the most severe chronic erosive joint disease (20). Our aim was to prepare cell wall fragments which, on the basis of past studies of the group A streptococcal PG-PS, would be most likely to induce chronic arthritis. PG-PS polymers were purified from two strains of *Streptococcus faecium* (group D), which is commonly isolated from human feces (44), and the anaerobes *Peptostreptococcus productus*, one of the 10 bacterial species most commonly isolated from human feces (23), and *Propionibacterium acnes*, a common isolate from human skin (30) and feces (23). The chemical compositions, in vitro lysozyme sensitivities, and arthropathic activities of these various cell wall polymers are described in this report.

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MATERIALS AND METHODS

Bacterial strains. The following bacterial strains were used: *Streptococcus pyogenes* (group A, type 3) strain D-58, *S. faecium* (group D) strain F-24, *S. faecium* (group D) strain ATCC 9790 (obtained from G. D. Shockman, Temple University School of Medicine, Philadelphia, Pa.), *Peptostreptococcus productus* VPI C18-23 (obtained from the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.), *Propionibacterium acnes* VPI 0009 (obtained from C. S. Cummins, Virginia Polytechnic Institute and State University).

In addition, *Methanobacterium formicicum* JF-1 was obtained as a frozen pellet of partially disintegrated whole cells from J. G. Ferry, Virginia Polytechnic Institute and State University. These bacteria were grown under strict anaerobic conditions with formate as the sole energy source, as previously described (34). After harvest by continuous-flow centrifugation, whole cells were disrupted by passage through a French pressure cell. The eluate was centrifuged at 10,000 × *g* for 20 min, and the pellet was frozen to −70°C, sent to us on dry ice, and maintained at −70°C until use in cell wall purification.

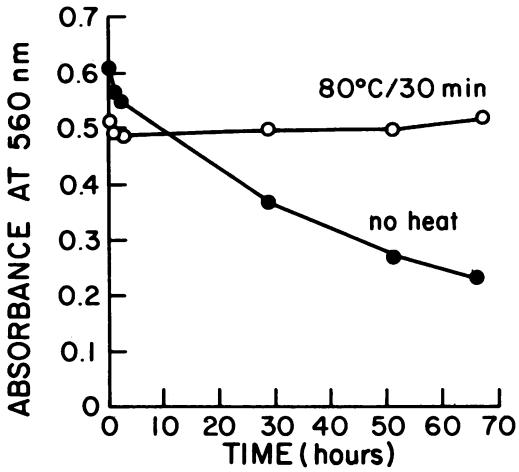


FIG. 1. Inactivation of the autolytic activity of *S. faecium* group D ATCC 9790 whole cells by heating at 80°C for 30 min. Heated and nonheated control cells were suspended in PBS at 37°C, and lysis was measured at A_{560}.

Cell wall purification. All bacterial strains, except *M. formicicum*, were grown to early-stationary phase in a 15-liter fermenter (The VirTis Co., Gardiner, N.Y.) at 37°C. During growth, additional glucose was added (1 liter of 45% glucose at 3 ml/min), and the pH was automatically maintained at 7.0 (6.5 for *Peptostreptococcus productus*) by the addition of 10% NaOH, essentially as described by Fox et al. (20). Streptococci were grown aerobically in Todd-Hewitt broth (BBL Microbiological Systems, Cockeysville, Md.) supplemented with Tween 80 (0.02%). *P. acnes* was grown anaerobically in Todd-Hewitt broth supplemented with the reducing agents sodium formaldehyde sulfoxylate (Eastman Kodak Co., Rochester, N.Y.) and cysteine (both at 0.03%) and Tween 80 (0.02%). *Peptostreptococcus productus* was grown anaerobically in brain heart infusion broth (BBL Microbiological Systems) supplemented with yeast extract (0.9%; Difco Laboratories, Detroit, Mich.), in addition to the concentrations of reducing agents and Tween 80 indicated above. All bacteria were harvested and cell walls were prepared in a similar manner as follows. To reduce autolytic and other metabolic activity, the culture was rapidly cooled to 4°C by passing −70°C ethanol through the heat exchange pipes of the fermenter. Bacteria were then harvested by continuous-flow centrifugation at 4°C. The pellet was suspended in cold phosphate-buffered saline (PBS), rapidly heated to 80°C by swirling in a boiling water bath, and maintained at 80°C for 30 min to inactivate autolytic enzymes (6, 40, Fig. 1). Bacteria were then washed three times with PBS, resuspended in PBS, and broken by shaking with glass beads in a Braun MSK cell homogenizer (Bronwill Scientific Inc., Rochester, N.Y.) cooled with liquid carbon dioxide. The partially disintegrated cells of *M. formicicum* were also briefly shaken with glass beads for more complete disruption. Cell walls (including those from *M. formicicum*) were separated from glass beads by filtration through a coarse-sintered glass filter, and the filtrate was centrifuged at 30,000 × *g* for 30 min. The resulting pellet consisted of a small hard-packed residuum of whole cells covered by a much larger, less dense layer of crude cell walls. The cell wall portion was suspended in approximately four times its packed volume in 2% sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, Mo.) and extracted for 2 h at 56°C. This extraction was repeated twice. Hot sodium dodecyl sulfate extractions were used to inactivate any residual autolytic activity and to remove contaminating lipid, protein, and nucleic acid (40). After each centrifugation, any remaining small pellet of whole cells was discarded. Cell walls were washed extensively (at least 10 times) with PBS at room temperature. After approximately the fourth wash, supernatants contained less than 0.003% sodium dodecyl sulfate as judged by the lack of a precipitate with 4 M KCl. Walls were then washed three times with distilled water, dialyzed against several changes of distilled water for at least 5 days, and lyophilized. This entire procedure was carried out under aseptic conditions, and the purified cell wall preparations were sterile as judged by the lack of growth on blood agar plates.

Cell wall composition. Neutral sugars and amino sugars were liberated by hydrolysis of purified cell walls in vacuo for 2 h in 2 N sulfuric acid at 100°C, converted to their alditol acetates, and analyzed by gas-liquid chromatography with xylose and methylglucamine (Sigma Chemical Co., St. Louis, Mo.) as internal standards, as previously described (21). Rhamnose was also determined colorimetrically by the method of Dische and Shettles (18). Prior to hydrolysis, cell walls from *P. acnes* and *Peptostreptococcus productus* were N acetylated by the method of Heymann et al. (22). This greatly increased the yield of muramic acid and glucosamine from these walls (25).

Amino acids were liberated by hydrolysis of purified cell walls for 18 h in vacuo in 6 N HCl at 110°C and quantitated on a high-pressure liquid chromatography-amino acid analyzer (Waters Associates, Inc., Milford, Mass.) (10).

Preparation and fractionation of cell wall fragments. Purified cell walls from each bacterial strain were sonicated and fractionated in an identical manner. Lyophilized cell walls were suspended in PBS at a concentration of 20 mg/ml, placed in a 20-ml stainless steel cup, and sonicated with cooling for 35 min, followed by a 15-min cooling period and another 35-min sonication, in a Branson model 350 Sonifer (Branson Sonic Power Co., Danbury, Conn.). Recently, we have found that a single 35-min sonication is sufficient to generate a wide range of fragment sizes from cell walls prepared by the sodium dodecyl sulfate extraction method. Therefore, in the experiment depicted in Fig. 5, rats were injected with fragments derived from a 35-min instead of 70-min sonication of cell walls. The sonically treated materials were centrifuged at 10,000 × *g* for 30 min to yield pellet and supernatant fractions. The supernatant fraction (10,000 × *g*) was further centrifuged at 100,000 × *g* for 60 min to yield pellet (100P) and supernatant fractions. The average molecular weight of the predominant group A streptococcal cell wall fragments in these fractions has been determined to be 500×10^6 (10,000 × *g* pellet), 50×10^6 (100P), and 5×10^6 (100,000 × *g* supernatant) (20).

Experimental animals and induction of arthritis. Outbred female Sprague-Dawley rats 6 to 8 weeks old were obtained from Zivic-Miller Laboratories, Allison Park, Pa. Eight to ten rats were used per group. Arthritis was induced by injection of sterile suspensions of cell wall fragments in PBS by either the intra-articular (i.a.) or i.p. route. Immediately prior to injection, each suspension was sonicated for 3 min to evenly disperse the fragments. The dose and fraction of fragments used are indicated in the Results. For i.a. injection, 10 μl of a suspension was injected into the right ankle of each rat through the Achilles tendon just above the calcaneus with a 25-gauge needle adapted to a micropipette (R. E. Esser, S. A. Stimpson, W. J. Cromartie, and J. H. Schwab, Arthr. Rheum., in press). Left ankle joints received

TABLE 1. Cell wall composition: sugars and amino sugars

Organism	μmol of sugar or amino sugar/mg (% [dry wt])						
	Rhamnose	Mannose	Galactose	Glucose	Muramic acid	Glucos-amine	Galactos-amine
Streptococcus pyogenes (group A, type 3)	1.9 (31.5)	0[a] (0)	0 (0)	0 (0)	0.17 (4.3)	1.2 (21.0)	0 (0)
Streptococcus faecium ATCC 9790	1.1 (18.6)	0 (0)	0 (0)	1.0 (18.7)	0.44 (11.0)	0.47 (8.4)	0.20 (3.6)
Streptococcus faecium F-24	1.1 (18.1)	0 (0)	0.46 (8.2)	0.75 (13.5)	0.31 (7.9)	0.26 (4.6)	0.20 (3.6)
Peptostreptococcus productus VPI C18-23	1.7 (27.5)	0 (0)	0.34 (6.2)	0.28 (5.0)	0.22 (5.6)	0.29 (5.2)	0.59 (10.6)
Propionibacterium acnes VPI 0009[b]	0 (0)	0.17 (3.0)	0.29 (5.3)	0.33 (6.0)	0.31 (7.7)	0.39 (7.0)	0.05 (0.9)
Methanobacterium formicicum JF-1	1.6 (25.8)	0.18 (3.2)	0.39 (7.0)	0 (0)	0 (0)	0.68 (12.2)	0.5 (9.0)

[a] A value of zero indicates that the component was not detected by our methods. No ribose, deoxyribose, or fucose was found in any of the cell wall preparations.
[b] *P. acnes* cell wall PS also contains 3 to 5% 2,3-diamino-2,3-dideoxyglucuronic acid (16).

10 μl of sterile PBS alone. For i.p. injection, 0.5 ml of suspension was injected per rat, and control groups received 0.5 ml of sterile PBS alone.

The severity of inflammation in each extremity was scored grossly on a scale of 0 (no apparent inflammation) to 4 (severe inflammation) based on erythema and edema of the periarticular tissues and enlargement of the joints, as previously described (13). The maximum score possible after an i.a. injection was 4 (inflammation was noted only in the ankle injected with cell wall polymers), and it was 16 after an i.p. injection (inflammation was assessed in the joints of each extremity distal to the knees and elbows).

After the i.p. injection of group A streptococcal cell wall fragments, it was our experience that an occasional rat never develops gross evidence of joint inflammation. However, the responses of all rats are included in the statistical calculations, even though some of these negative responses are probably due to inadvertent injection into the intestine.

Histology. In the first experiment, groups of rats were injected with group A or group D (ATCC 9790 or F-24) streptococcal cell wall fragments (all at 130 μg [dry weight]/g of body weight) or PBS alone. On days 3, 30, and 60 after injection, three rats from each group were sacrificed. In the second experiment, which focused on a comparison of the chronic phase of disease (see Fig. 5), all rats were sacrificed on day 128. Immediately after sacrifice, skinned ankle joints were removed, fixed in 10% Formalin, and decalcified in EDTA. Paraffin sections were then prepared and stained with Weigart hematoxylin and alcoholic eosin (13).

Radiology. Rats were anesthetized with an intramuscular injection of Innovar (droperidol-fentanyl citrate; McNeil Laboratories, Inc., Ft. Washington, Pa.), and ankles were radiographed on Kodak projector slide plates (100 to 200 line pairs per mm) with a microradiographic unit (Faxitron model 43804; Hewlett-Packard Co., McMinnville, Oreg.). The radiographs were evaluated for evidence of the presence and severity of soft tissue swelling, demineralization, erosions, subperiosteal new bone, joint space narrowing, degenerative changes, and alignment alterations by a grading system previously described (9).

RESULTS

Cell wall composition. The sugar, amino sugar, and amino acid composition of cell walls from the various bacterial strains used in this study are shown in Tables 1 and 2. The results indicate that all are complexes of PG, or pseudomurein in the case of *M. formicicum*, and PS. These analyses are consistent with current knowledge of the structure of cell walls from these bacteria. Four different PG structures are represented here. All contain L-alanine and D-glutamic acid in the first two positions of the peptide but differ in the type of diamino acid and the nature of the cross-linkage (interpeptide bridge). Group A streptococcal PG contains lysine and is cross-linked with a dipeptide of L-alanine (35). *S. faecium* (group D) PG contains lysine and is cross-linked with a single residue of D-isoasparagine (27). *Peptostreptococcus productus* PG contains *meso*-diaminopimelic acid and is directly cross-linked (46). *P. acnes* VPI 0009 PG contains L,L-diaminopimelic acid (14), and although glycine is present as either an N- or C-terminal residue on L,L-

TABLE 2. Cell wall composition: amino acids

Organism	Molar ratio[a] of amino acid (% [dry wt])					
	Aspartic acid	Glutamic acid	Glycine	Alanine	Lysine	Diamino-pimelic acid
Streptococcus pyogenes (group A, type 3)	0 (—)[b]	1.1 (7.5)	0 (—)	2.9 (11.5)	1.0 (6.6)	0 (—)
Streptococcus faecium ATCC 9790	1.1 (5.1)	1.3 (6.5)	0 (—)	2.0 (6.1)	1.0 (5.1)	0 (—)
Streptococcus faecium F-24	0.9 (3.7)	1.1 (5.2)	0 (—)	1.7 (4.8)	1.0 (4.7)	0 (—)
Peptostreptococcus productus VPI C18-23	0 (—)	0.9 (5.5)	0 (—)	2.2 (8.2)	0 (—)	1.0 (8.0)
Propionibacterium acnes VPI 0009	0 (—)	0.8 (8.2)	0.8 (4.1)	1.7 (10.7)	0 (—)	1.0 (13.3)
Methanobacterium formicicum JF-1	0 (—)	2 (12.5)	0 (—)	1.3 (4.9)	1.0 (6.3)	0 (—)

[a] For the molar ratio, lysine or diaminopimelic acid was 1.0.
[b] —, Amino acid was either not found or detected only in trace amounts (less than 0.06 μmol/mg).

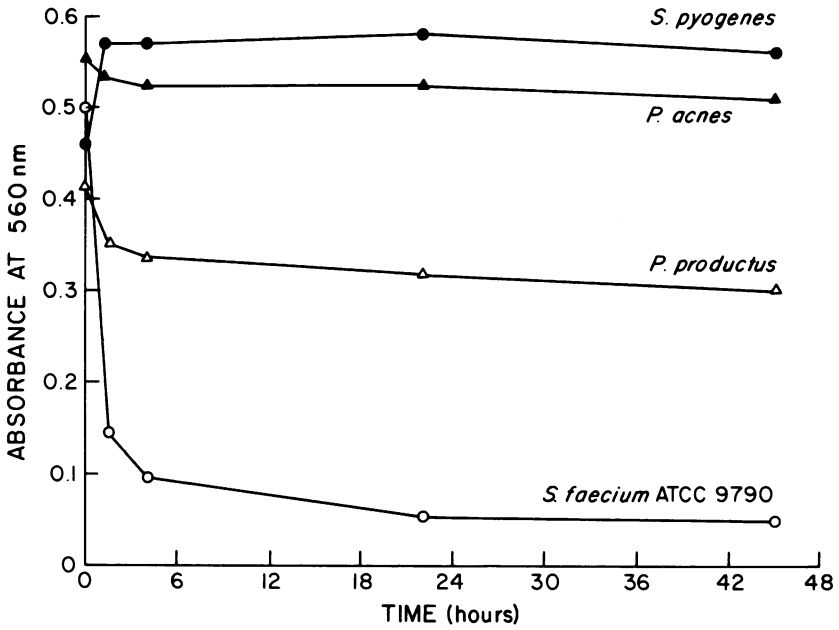


FIG. 2. Lysis of various PG-PS polymers by hen egg white lysozyme. Purified, unsonicated PG-PS polymers from various bacteria were suspended at approximately 1 mg/ml in 25 mM sodium phosphate buffer with 0.2% NaCl (pH 7.0) diluted at time zero with an equal volume of buffer containing hen egg white lysozyme (400 μg/ml) and incubated at 37°C.

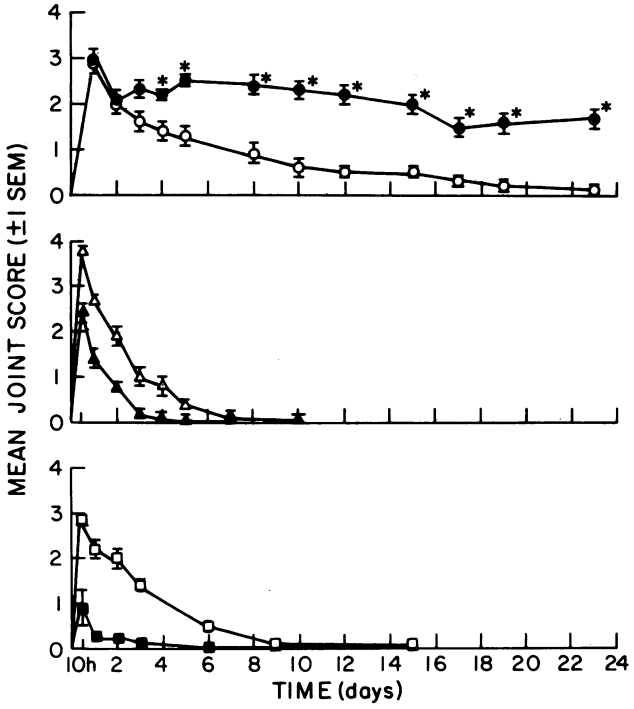


FIG. 3. Inflammation induced in rat ankle joints by the i.a. injection of PG-PS 100P fragments isolated from group A and group D streptococci, *Peptostreptococcus productus*, and *P. acnes* and pseudomurein-PS fragments from *M. formicicum*. The dose indicated was injected into the right ankle of each rat. Left ankles received PBS alone and did not become inflamed (data not shown). Joint inflammation induced by group A streptococcal PG-PS was significantly more severe than that induced by group D streptococcal PG-PS on the days indicated by asterisks (12 rats per group, $P < 0.001$). Symbols: ●, 10 μg of *S. pyogenes* (group A) PG-PS; ○, 10 μg of *S. faecium* (group D) ATCC 9790 PG-PS; ▲, 10 μg of *P. acnes* PG-PS; △, 10 μg of *Peptostreptococcus productus* PG-PS; ■, 10 μg of *M. formicicum* pseudomurein-PS; □, 50 μg of *M. formicicum* pseudomurein-PS.

diaminopimelic acid, recent reports indicate that this PG is directly cross-linked (24, 25). *M. formicicum* cell walls contain pseudomurein instead of the conventional PG structure, which has L-*N*-acetyltalosaminuronic acid instead of D-*N*-acetylmuramic acid, and also contains no D-amino acids (26, 28). Although *N*-acetyltalosaminuronic acid was not measured here, we found no trace of muramic acid, and the molar ratios of the amino acids are consistent with that previously reported (26).

Only the PS from group A streptococci has been studied in detail, and our compositional analyses are consistent with the current knowledge of this structure (11). The grouping polysaccharide of group D streptococci is a membrane component (39), and antigenic differences in the cell wall (type specific) PS are known to exist among different strains in this group (6). The only major difference we found in the composition of cell walls from the F-24 and ATCC 9790 strains of *S. faecium* was the presence of galactose in F-24. Recent studies on the penicillin-induced secretion of high-molecular-weight cell wall polymers from autolytic-defective mutants of ATCC 9790 suggest that *S. faecium* PG could be substituted with at least two different types of PS structures (R. Kariyama, D. L. Dolinger, and G. D. Shockman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K85, p. 185).

The purified cell wall polymers used in this study are referred to as PG-PS or pseudomurein-PS polymers to distinguish them from crude or native cell walls which contain proteins, nucleic acids, and other cellular components.

Lysozyme sensitivity of purified cell wall polymers. The

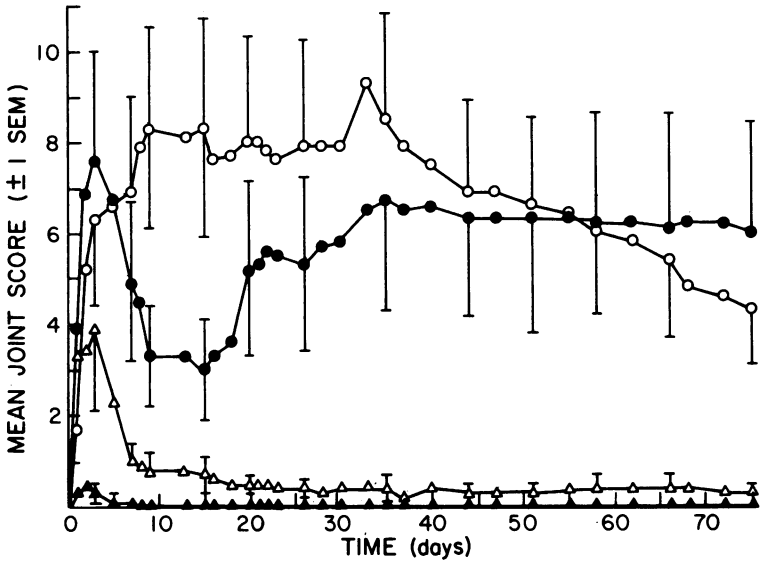


FIG. 4. Arthritis induced by i.p. injection of various PG-PS 100P fragments into rats. Dosages: *S. pyogenes* (group A), 130 μg [dry weight]/g of body weight; *S. faecium* (group D) ATCC 9790, 200 μg [dry weight]/g of body weight; *Peptostreptococcus productus*, 200 μg [dry weight]/g of body weight; and *P. acnes*, 200 μg [dry weight]/g of body weight. A negative control group which received PBS alone never developed arthritis. For clarity, standard errors are not indicated for every data point. Symbols: ●, *S. pyogenes*; ○, *S. faecium* ATCC 9790; △, *Peptostreptococcus productus*; ▲, *P. acnes*.

sensitivity of the various PG-PS polymers to hen egg white lysozyme is shown in Fig. 2. We confirm the findings that PG-PS polymers from *P. acnes* (15) and *S. pyogenes* (37) are not sensitive, while those from *S. faecium* ATCC 9790 (1, 41) are sensitive to lysozyme. *Peptostreptococcus productus* PG-PS had an intermediate sensitivity to lysozyme. Other studies indicated that the lysozyme sensitivities of PG-PS from group D streptococcal strains ATCC 9790 and F-24 did not differ, that pseudomurein-PS polymers from *M. formicicum* were not lysed by lysozyme and that all purified cell wall polymer preparations were stable as judged by the lack of detectable lysis in PBS alone (data not shown).

Joint inflammation induced by i.a. injection of cell wall polymers. Figure 3 shows the course of arthritis induced by a single i.a. injection of the 100P fragments of the various cell wall polymers. All induced an acute inflammation which peaked by 12 to 24 h and gradually subsided. *S. pyogenes* (group A) PG-PS induced the most severe, prolonged inflammation, which was significantly more severe than that induced by the same dose of *S. faecium* (group D) PG-PS from day 4 to the termination of the experiment. Of the PG-PS fragments tested, that from *P. acnes* induced the least severe inflammation. Pseudomurein-PS fragments from *M. formicicum* were less active than any of the PG-PS preparations. Approximately five times more pseudomurein-PS was required to induce an acute inflammation equal in severity to that induced by PG-PS fragments.

Arthritis induced by i.p. injection of cell wall polymers. Figure 4 depicts the course of arthritis induced by a single i.p. injection of the 100P fragments of various cell wall polymers. Group A streptococcal PG-PS, as described in detail previously (13), induced an acute arthritis peaking at day 3, followed by a partial resolution and then recurrence of inflammation by 3 weeks after injection. Repeated episodes of remission and exacerbation of arthritis continued throughout the chronic phase of disease, and severe arthritis was still grossly evident when the experiment was terminated on day 75. Group D streptococcal ATCC 9790 PG-PS induced a severe acute arthritis peaking about 1 week after injection which did not begin to decrease in severity until 5 weeks later. At the termination of the experiment, arthritis appeared to be resolving, in contrast to the ongoing joint disease seen in group A streptococcal PG-PS-injected rats. This observation prompted the detailed comparison of group A and group D streptococcal PG-PS-induced arthritis in the long-term experiment described below. *Peptostreptococcus productus* PG-PS induced a moderately severe acute arthritis which peaked at day 3, followed by a mild chronic disease in two of the eight rats injected. *P. acnes* PG-PS induced very little acute and no chronic arthritis upon i.p. injection. No arthritis was seen in negative control rats injected with PBS alone.

In other experiments (data not shown), the i.p. injection of *M. formicicum* pseudomurein-PS 100P fragments (250 μg [dry weight]/g of body weight, 4 rats, 30-day observation period) or 10,000 × *g*-supernatant fragments (250 μg [dry weight]/g of body weight, 8 rats, 42-day observation period) did not induce any gross evidence of joint inflammation or other toxic effects, such as the transient inactivity of the animals which often follows the i.p. injection of a similar dose of PG-PS fragments.

Comparison of arthritis induced by i.p. injection of PG-PS fragments from group A and group D streptococci. The gross, histologic, radiologic, and recurrent features of arthritis induced by PG-PS 100P fragments isolated from group A and two strains of group D streptococci were compared. Figure 5 shows the course of arthritis as judged by gross inspection of the joints. All three preparations induced an acute arthritis which peaked about day 4. Group A streptococcal PG-PS induced a typical response, with partial resolution of the acute reaction, followed by a recurrence of inflammation 3 weeks later, which evolved into a chronic irreversible joint disease. The mean severity of this chronic phase remained at a high level for the duration of the experiment, and the phase was characterized by repeated episodes of remission and exacerbation in individual joints (Table 3). Ankylosis of many joints became grossly apparent after approximately 2 months. Acute arthritis induced by the two group D strep-

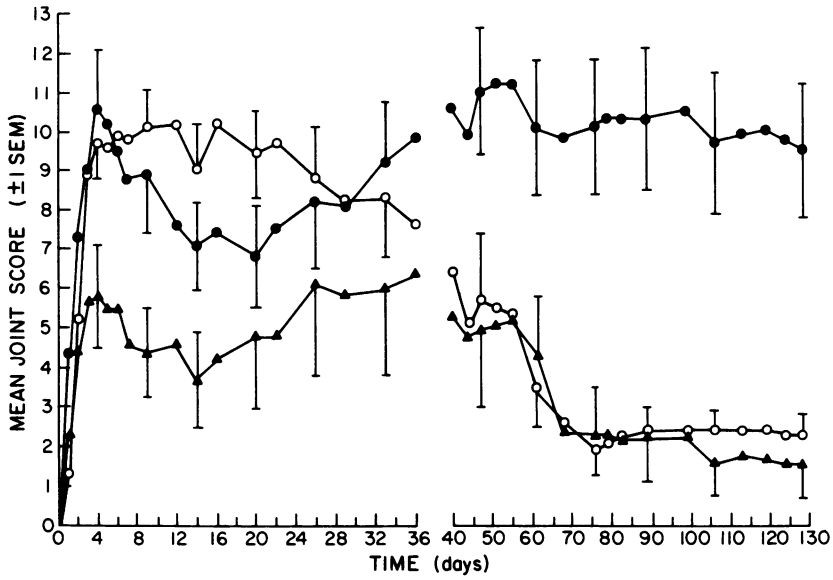


FIG. 5. Arthritis induced in rats by the i.p. injection of PG-PS fragments (150 μg [dry weight]/g of body weight) isolated from *S. pyogenes* (group A) (●), *S. faecium* (group D) ATCC 9790 (○), and *S. faecium* (group D) F-24 (▲). Ten rats were injected in each test group. A negative control group which received PBS alone never developed arthritis (data not shown). The level of significance between the three groups was calculated for each day that scores were recorded. For clarity, standard errors are not indicated for every data point. Arthritis was more severe in group A than group D (either strain) PG-PS-injected rats on days 40 to 61 ($P < 0.05$) and 68 to 128 ($P < 0.001$). Other statistical comparisons are indicated in the text.

tococcal preparations also evolved into a chronic phase, but in contrast to that induced by group A streptococcal PG-PS, significantly fewer episodes of recurrent inflammation were observed (Table 3), and grossly apparent inflammation began to resolve after about 35 days. After day 80, joints no longer had the erythematous, warm, inflamed appearance of joints from group A streptococcal PG-PS-injected rats, and little ankylosis was noted.

The patterns of arthritis induced by PG-PS fragments from the two group D streptococcal strains were not identical (Fig. 5). The ATCC 9790 PG-PS induced an acute and early chronic reaction which was significantly more severe than that induced by F-24 PG-PS on days 3 to 22 ($P < 0.05$).

Gross observations of joint injury induced by group A and group D streptococcal PG-PS fragments were confirmed by radiologic and histologic studies. The radiologic features of group A streptococcal PG-PS-induced arthritis were similar to those described in detail previously (9). Radiographs taken on day 30 of ankle joints prior to the sacrifice of rats for histologic study revealed no major differences. All joints received a total radiologic score of 2 to 5 due to various degrees of demineralization, periostitis, and soft tissue swelling. No radiologic evidence of erosions was seen at 30 days (data not shown). Radiographs taken on day 110 of the ankle joints of rats sacrificed for the day-128 histology study revealed that the radiologic score for group A rats was significantly higher than that for group D rats (Table 3). Scores for rats receiving group D streptococci ATCC 9790 PG-PS were similar to those for rats receiving group D streptococcal F-24 PG-PS, with clear evidence of demineralization, periostitis, and soft tissue swelling. However, in constrast to ankles from group A streptococcal PG-PS-injected rats, little or no evidence of erosions or cartilage space narrowing was noted.

Histologic studies of joint tissue from rats sacrificed 3 days after i.p. injection of group A or either strain of group D streptococcal PG-PS revealed similar extensive acute exudative reactions consisting of vascular congestion, evidence of edema, fibrin deposits, infiltration by neutrophils and mononuclear cells, and extravasation of erythrocytes. The changes were most extensive in the stroma and synovial membrane but also extended into the joint capsule and periarticular tissues (Fig. 6a and b). Tissues collected 30, 60, and 128 days after injection of group A streptococcal PG-PS showed that the inflammatory process evolved into an erosive destructive arthritis as previously described (13). The types of changes observed are illustrated in Fig. 6c and e.

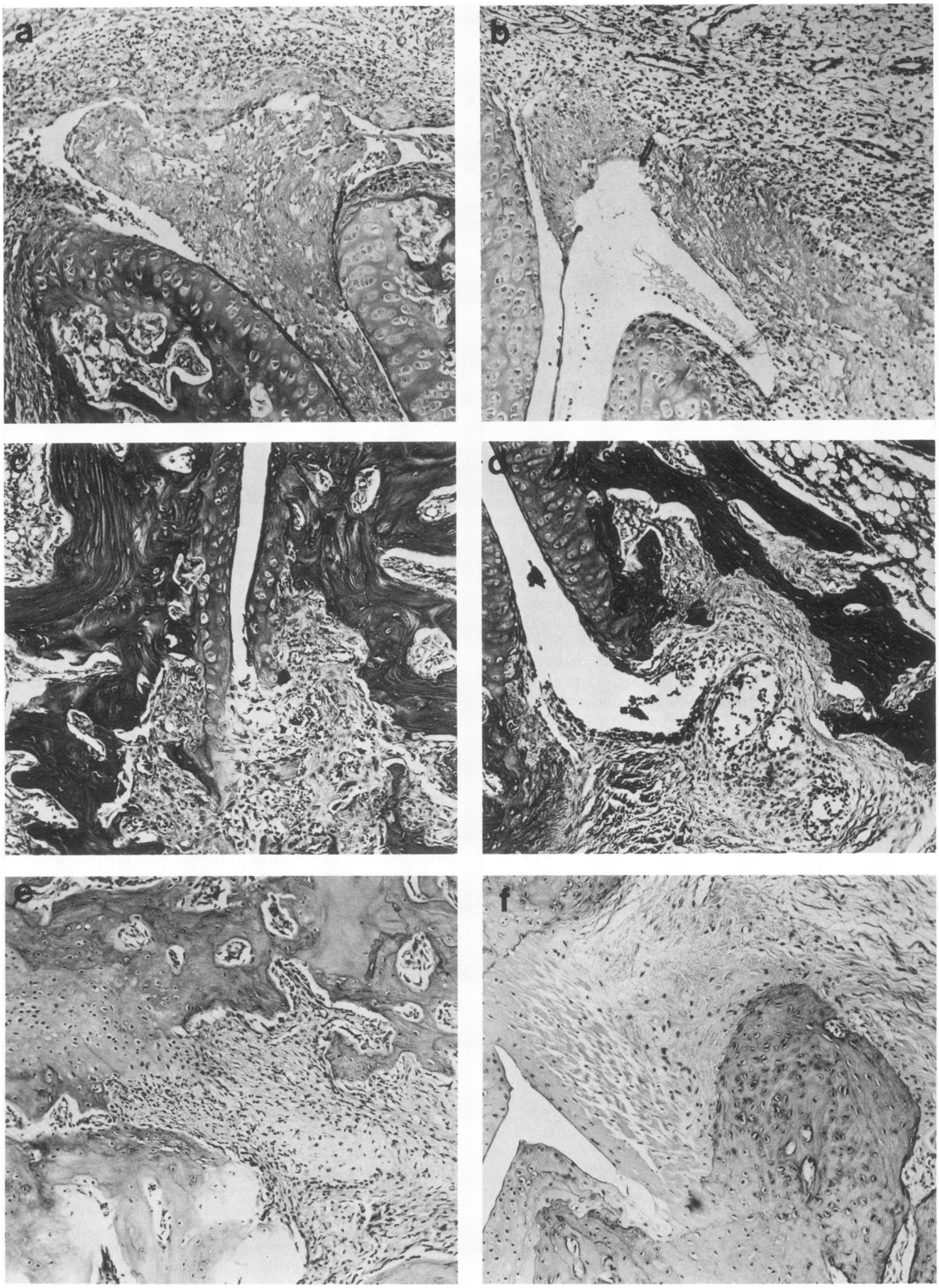
TABLE 3. Comparison of the recurrent and radiologic features of arthritis induced by i.p. injection of *S. pyogenes* or *S. faecium* PG-PS fragments[a]

PG-PS source	Mean no. of exacerbations/rat ± SEM[b]	Mean radiologic score ± SEM[c]
S. pyogenes (group A)	6.7 ± 1.2	5.6 ± 1.3
S. faecium (group D) ATCC 9790	2.8 ± 0.8 ($P < 0.01$)	1.7 ± 0.3 ($P < 0.005$)
S. faecium (group D) F-24	2.2 ± 0.8 ($P < 0.005$)	1.3 ± 0.5 ($P < 0.005$)

[a] Data in this table are based on the experiment depicted in Fig. 5. Significant difference is given for *S. pyogenes* PG-PS-injected versus *S. faecium* PG-PS-injected rats.

[b] The course of arthritis in each ankle and wrist joint of each rat was examined for spontaneous exacerbations of grossly apparent inflammation. A successive decrease and increase of at least 1 joint score unit was considered an exacerbation. The number of exacerbations observed in each of the four joints was summed and expressed as the number of exacerbations per rat.

[c] On day 110 after PG-PS injection, the ankle joints were analyzed for radiologic evidence of arthritis. See Materials and Methods for details of the radiologic evaluation.


a
b
c
e
f

Evidence of cartilage and bone erosion and chronic inflammation was present in joints collected as late as 128 days after injection. Tissues collected 30 and 60 days after injection of group D streptococcal F-24 PG-PS showed erosive arthritis similar to that induced by group A streptococcal PG-PS; however, the extent of the erosion of cartilage and adjacent bone was less than that induced by group A streptococcal PG-PS (Fig. 6d). The sections from rats sacrificed 30 and 60 days after injection of group D streptococcal ATCC 9790 PG-PS showed a moderately severe chronic synovitis without evidence of erosion of cartilage and adjacent bone. The joints collected 128 days after injection of PG-PS from either group D streptococcal strain showed histologic evidence of previous injury, consisting of new bone formation and fibrosis of the synovial stroma and periarticular tissues, without evidence of active chronic inflammation (Fig. 6f).

DISCUSSION

These studies show that a sterile, aqueous suspension of PG-PS polymers from normal human flora can induce a wide range of arthropathic responses upon i.p. injection into rats. The pathology varies from a minimal acute arthritis (*P. acnes*), to a moderately severe acute reaction and mild chronic disease (*Peptostreptococcus productus*), to a severe acute and chronic erosive arthritis (group D streptococci). While group D streptococcal PG-PS clearly induced chronic arthritis, it is of relatively short duration, leaving the joints slightly enlarged, with histologic evidence of residual tissue damage but without active, progressive chronic inflammation. The group B streptococcal cell wall fragments studied by Spitznagel et al. (43) also induced chronic arthritis of limited duration. Group A streptococcal PG-PS is the only type we have worked with to date which has induced chronic erosive arthritis of a recurrent irreversible nature leading to joint destruction. *L. casei* cell wall fragments induced chronic erosive arthritis of at least 60 days duration, but whether this arthritis has the extraordinary long-term, recurrent, irreversible features of group A streptococcal PG-PS-induced arthritis cannot be determined from the relatively short-term experiments reported by Lehman et al. (29).

Previous reports have indicated that sonically treated group D streptococcal whole cells induced little or no arthritis, and group D streptococcal cell walls induced only a transient acute joint inflammation (12, 13, 43). The determination of what new aspect of purification (i.e., inactivation of autolytic activity or preparation of the appropriate fragment size) was most important in rendering group D streptococcal PG-PS able to induce chronic erosive arthritis in the present study will require more work. In any case, the new preparation probably more accurately reflects the intrinsic arthropathic potential of group D streptococcal PG-PS.

Based on studies of the group A streptococcal PG-PS, the major bacterial determinants of arthropathic activity include the ability of the PG moiety to induce acute inflammation (20) and the capacity of the PG-PS complex to persist in tissue, which is correlated with resistance to hen egg white lysozyme in vitro (17, 19, 32, 42). The nature of the joint inflammation is also related to the molecular size of the PG-PS complex (8, 20). The isolated PS moiety (7) and low-molecular-weight PG-PS fragments (8) induce a transient edematous reponse in joint tissue upon systemic injection; this change in vascular permeability may be an important event in the localization of PG-PS fragments to the joints. In addition, since the removal of PS renders PG sensitive to lysozyme, the PS contributes to the lysozyme resistance of the PG-PS complex (37).

The inactivity upon intravenous injection and relative inactivity upon i.a. injection of pseudomurein-PS fragments from *M. formicicum* is consistent with a crucial role for the PG moiety of the PG-PS. During purification, pseudomurein-PS fragments behaved in a manner very similar to that of conventional PG-PS, suggesting that their physical properties, such as size, sensitivity to sonic disruption, and sedimentation characteristics, are similar to those of PG-PS fragments. Thus, this nonarthropathic analog of PG-PS will be useful as a control in future experiments. It will also be of interest to determine whether the isolated pseudomurein moiety has any of the biological activities, such as complement activation and adjuvanticity, of conventional PG. All of the PG-PS types induced an acute inflammation upon i.a. injection. The relative order of the severity and duration of joint inflammation induced by the various PG-PS types mimicked that seen in response to an i.p. injection.

The in vitro action of hen egg white lysozyme on the PG-PS types studied here did not in itself predict ability to induce chronic arthritis. However, the in vitro reaction with hen egg white lysozyme may not represent the nature and extent of PG-PS degradation caused by rat lysozyme and peptidases in the in vivo milieu. The relationship between PG-PS degradation and arthropathic activity is being pursued in our laboratory by studying the in vivo degradation of radiolabeled PG-PS fragments in rats.

With the exception of the group-specific PS of group A streptococcal PG-PS, the detailed structures and biological activities of the PS components of the various PG-PS complexes are not known. It is intriguing that all of the PG-PS polymers which induce chronic arthritis have in common rhamnose as a major PS residue. However, given the short list of PG-PS types thus far examined and the lack of information on precise structural details, it would be premature to assign a crucial role to any specific PS structure or component. The biological activities of the various PS polymers and their abilities to influence the persistence and other properties of the PG moiety will be investigated in future studies.

P. acnes whole cells and cell walls have been the subject of many investigations due to interest in the antitumor and immunomodulatory activities of this organism (also known

FIG. 6. Histologic appearance of ankle joints prepared from rats sacrificed at intervals after the i.p. injection of group A or group D (F-24) streptococcal PG-PS (magnification, ×120 for all photomicrographs). (a) Acute exudative reaction 3 days after group A streptococcal PG-PS injection. A feature of the reaction is the presence of a large amount of fibrin in the joint space. (b) Acute exudative reaction, similar to that induced by group A streptococcal PG-PS, 3 days after group D streptococcal PG-PS injection. (c) Chronic erosive synovitis 30 days after group A streptococcal PG-PS injection. The destroyed cartilage and adjacent bone has been replaced by pannus consisting of capillaries and hyperplastic fibrous tissue, infiltrated by mononuclear cells. (d) Chronic erosive arthritis, similar to that seen in response to group A streptococcal PG-PS, 30 days after group D streptococcal PG-PS injection. (e) Arthritis 128 days after group A streptococcal PG-PS injection, showing destruction of the cartilage and subchondral bone and replacement by fibrous tissue infiltrated with mononuclear cells. (f) Residual joint damage 128 days after group D streptococcal PG-PS injection, showing fibrosis of the synovial tissue and ectopic formation of new bone.

as *Corynebacterium parvum*, reviewed in reference 31). Whole cells persist for long periods in vivo (38) and in macrophages in vitro (33), which correlates with the in vitro resistance of the PG-PS to lysozyme digestion. *P. acnes* cell walls share other properties with group A streptococcal PG-PS, including adjuvanticity (3, 24), mitogenicity (4), and activation of the alternate complement pathway (45). In contrast to group A streptococcal PG, Webster et al. (45) found that *P. acnes* PG was not responsible for the complement-activating activity of cell walls. Also, a systemic injection of *P. acnes* PG was unable to induce a recurrence of inflammation in joints given an i.a. injection of group A streptococcal PG-PS 3 weeks earlier, whereas group A streptococcal PG and group D streptococcal PG-PS were both highly active in this model of recurrent inflammation (R. E. Esser et al., Arth. Rheum., in press). These observations, considered with the relatively mild inflammation induced by *P. acnes* PG-PS upon i.a. injection, suggest that the capacity of *P. acnes* PG to induce acute inflammation might reflect the reportedly unique structure of the peptide region of *P. acnes* PG (24, 25) and might be responsible for the weak arthropathic activity of *P. acnes* PG-PS upon i.p. injection in rats. The arthropathic activity of *P. acnes* PG-PS in this model is of particular interest since a high-molecular-weight antigen which cross-reacts with a *P. acnes* PS antigen has been reported to be extracted from synovial fluid and synovial leukocytes from patients with rheumatoid arthritis (5). Future experiments will examine the inflammatory and other properties of the isolated PG structures of the various PG-PS polymers.

We hypothesize that the normal flora, particularly of the intestine, harbors a sufficiently diverse population of bacteria to provide PG-PS polymers of many different sizes and structures, capable of inducing a wide range of arthopathic responses in the host. The varied responses induced by the small representative group of polymers thus far studied support this hypothesis. We further hypothesize that arthropathic polymers might accumulate within the host over a long period as a natural consequence of repeated exposure to and uptake of certain albeit small amounts of normal flora bacteria or bacterial debris from infection or both. This process might be greatly accelerated when the intestinal epithelium is damaged, as in gastrointestinal disease. Arthritis might then result, in the susceptible host, from the accumulation of a certain threshold concentration of arthropathic polymers in joint tissue. Wensinck and van de Merwe (47) noted increased incidence of serum antibodies against intestinal anaerobes, including the strain of *Peptostreptococcus productus* used in our experiments, in Crohn's disease patients, suggesting increased exposure to indigenous intestinal bacteria. These studies, together with the known association of arthritis with Crohn's and other gastrointestinal diseases (2), are consistent with a role for arthropathic PG-PS polymers derived from intestinal bacteria in the etiology and pathogenesis of arthritis.

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LITERATURE CITED

1. **Abrams, A.** 1958. O-acetyl groups in the cell wall of *Streptococcus faecalis*. J. Biol. Chem. **230**:949–959.
2. **Ansell, B. M.** 1976. Arthritis in gastrointestinal disease, p. 129–132. *In* D. C. Dumonde (ed.), Infection and immunology in the rheumatic diseases. Blackwell Scientific Publications, Ltd., Oxford.
3. **Azuma, I., K. Sugimura, T. Taniyama, A. Aladin, and Y. Yamamura.** 1975. Chemical and immunological studies on the cell walls of *Propionibacterium acnes* strain C7 and *Corynebacterium parvum* ATCC 11829. Jpn. J. Microbiol. **19**:265–275.
4. **Azuma, I., T. Taniyama, K. Sugimura, A. A. Aladin, and Y. Yamamura.** 1976. Mitogenic activity of the cell walls of mycobacteria, *Nocardia*, corynebacteria, and anaerobic coryneforms. Jpn. J. Microbiol. **20**:263–271.
5. **Bartholomew, L., and F. N. Bartholomew.** 1979. Antigenic bacterial polysaccharide in rheumatoid synovial effusions. Arth. Rheum. **22**:969–977.
6. **Bleiweis, A. S., and R. M. Krause.** 1965. The cell walls of group D streptococci. I. The immunochemistry of the type I carbohydrate. J. Exp. Med. **122**:237–249.
7. **Chetty, C., R. R. Brown, and J. H. Schwab.** 1983. Edema-producing activity of group A streptococcal polysaccharide and its possible role in the pathogenesis of cell wall-induced polyarthritis. J. Exp. Med. **157**:1089–1100.
8. **Chetty, C., D. G. Klapper, and J. H. Schwab.** 1982. Soluble peptidoglycan-polysaccharide fragments of the bacterial cell wall induce acute inflammation. Infect. Immun. **38**:1010–1019.
9. **Clark, R. L., J. T. Cuttino, S. K. Anderle, W. J. Cromartie, and J. H. Schwab.** 1979. Radiologic analysis of arthritis in rats after systemic injection of streptococcal cell walls. Arthr. Rheum. **22**:25–35.
10. **Cohen, S. A., T. L. Tarvin, and B. A. Bidlingmeyer.** 1984. Analysis of amino acids using precolumn derivatization with phenylisothiocyanate. Am. Lab. **16**:48–59.
11. **Coligan, J. E., T. J. Kindt, and R. M. Krause.** 1978. Structure of the streptococcal groups A, A-variant, and C carbohydrates. Immunochemistry **15**:755–760.
12. **Cromartie, W. J.** 1981. Arthropathic properties of peptidoglycan-polysaccharide complexes of microbial origin, p. 24–38. *In* H. Deicher and L. C. Schulz (ed.), Arthritis models and mechanisms. Springer-Verlag KG, Berlin.
13. **Cromartie, W. J., J. G. Craddock, J. H. Schwab, S. K. Anderle, and C. Yang.** 1977. Arthritis in rats after systemic injection of streptococcal cells or cell walls. J. Exp. Med. **146**:1585–1602.
14. **Cummins, C. S., and J. L. Johnson.** 1974. *Corynebacterium parvum*: a synonym for *Propionibacterium acnes*? J. Gen. Microbiol. **80**:433–442.
15. **Cummins, C. S., S. A. Stimpson, R. L. Tuttle, and C. J. Weck.** 1981. Observations on the nature of the determinants in anaerobic coryneforms which produce splenomegaly. RES J. Reticuloendothel. Soc. **29**:1–13.
16. **Cummins, C. S., and R. H. White.** 1983. Isolation, identification, and synthesis of 2,3-diamino-2,3-dideoxyglucuronic acid: a component of *Propionibacterium acnes* cell wall polysaccharide. J. Bacteriol. **153**:1388–1393.
17. **Dalldorf, F. G., W. J. Cromartie, S. K. Anderle, R. L. Clark, and J. H. Schwab.** 1980. The relation of experimental arthritis to the distribution of streptococcal cell wall fragments. Am. J. Pathol. **100**:383–402.
18. **Dische, A., and L. B. Shettles.** 1948. A specific color reaction of methyl pentose and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.
19. **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.
20. **Fox, A., R. R. Brown, S. K. Anderle, C. Chetty, W. J. Cromartie, H. Gooder, and J. H. Schwab.** 1982. Arthropathic properties related to the molecular weight of peptidoglycan-polysaccharide polymers of streptococcal cell walls. Infect. Immun. **35**:1003–1010.
21. **Fox, A., J. H. Schwab, and T. Cochran.** 1980. Muramic acid detection in mammalian tissues by gas-liquid chromatography-

mass spectrometry. Infect. Immun. **29**:526–531.
22. **Heymann, H., J. M. Manniello, and S. S. Barkulis.** 1964. Structure of streptococcal cell walls. III. Characterization of an alanine-containing glucosaminylmuramic acid derivative liberated by lysozyme from streptococcal glycopeptide. J. Biol. Chem. **239**:2981–2985.
23. **Holdeman, L. V., I. J. Good, and W. E. C. Moore.** 1976. Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Appl. Environ. Microbiol. **31**:359–375.
24. **Kamisango, K., H. Fujii, Y. Yanagihara, I. Mifuchi, and I. Azuma.** 1983. Structures of peptidoglycans from *Propionibacterium*. Microbiol. Immunol. **27**:635–640.
25. **Kamisango, K., I. Saiki, Y. Tanio, H. Okumura, Y. Araki, I. Sekikawa, I. Azuma, and Y. Yamamura.** 1982. Structures and biological activities of peptidoglycans of *Listeria monocytogenes* and *Propionibacterium acnes*. J. Biochem. **92**:23–33.
26. **Kandler, O., and H. Konig.** 1978. Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch. Microbiol. **118**:141–152.
27. **Kandler, O., K. H. Schleifer, and R. Dandl.** 1968. Differentiation of *Streptococcus faecalis* Andrewes and Horder and *Streptococcus faecium* Orla-Jensen based on the amino acid composition of their murein. J. Bacteriol. **96**:1935–1939.
28. **Konig, H., and O. Kandler.** 1980. 2-Amino-2-deoxytaluronic acid and 2-amino-2-deoxyglucose from the pseudomurein of *Methanobacterium thermoautotrophicum* possess the L- and D-configurations. Hoppe-Seyler's Z. Physiol. Chem. **361**: 981– 983.
29. **Lehman, T. J. A., J. B. Allen, P. H. Plotz, and R. L. Wilder.** 1983. Polyarthritis in rats following the systemic injection of *Lactobacillus casei* cell walls in aqueous suspension. Arth. Rheum. **26**:1259–1265.
30. **McGinley, K. J., G. F. Webster, and J. J. Leyden.** 1978. Regional variations of cutaneous propionibacteria. Appl. Environ. Microbiol. **35**:62–66.
31. **Milas, L., and M. T. Scott.** 1978. Antitumor activity of *Corynebacterium parvum*. Adv. Cancer Res. **26**:257–306.
32. **Ohanian, S. H., and J. H. Schwab.** 1967. Persistence of group A streptococcal cell walls related to chronic inflammation of rabbit dermal connective tissue. J. Exp. Med. **125**:1137–1148.
33. **Pringle, A. T., C. S. Cummins, B. F. Bishop, and V. S. Viers.** 1982. Fate of vaccines of *Propionibacterium acnes* after phagocytosis by murine macrophages. Infect. Immun. **38**:371–374.
34. **Schauer, N. L., and J. G. Ferry.** 1982. Properties of formate dehydrogenase in *Methanobacterium formicicum*. J. Bacteriol. **150**:1–7.
35. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407–477.
36. **Schwab, J. H.** 1979. Acute and chronic inflammation induced by bacterial cell wall structures, p. 209–214. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
37. **Schwab, J. H., and S. H. Ohanian.** 1967. Degradation of streptococcal cell wall antigens in vivo. J. Bacteriol. **94**:1346–1352.
38. **Scott, M. T., and L. Milas.** 1977. Distribution and persistence *in vivo* of *Corynebacterium parvum* in relation to its antitumor activity. Cancer Res. **37**:1673–1679.
39. **Shockman, G. D., and H. D. Slade.** 1964. The cellular location of the streptococcal group D antigen. J. Gen. Microbiol. **37**:297–305.
40. **Shockman, G. D., J. S. Thompson, and M. J. Conover.** 1967. The autolytic enzyme system of *Streptococcus faecalis*. II. Partial characterization of the autolysin and its substrate. Biochemistry **6**:1054–1065.
41. **Shockman, G. D., J. S. Thompson, and M. J. Conover.** 1968. The relationship of autolysin to lysozyme sensitivity of *Streptococcus faecalis* 9790, p. 248–260. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts, and L-forms. The Williams & Wilkins Co., Baltimore.
42. **Smialowicz, R. J., and J. H. Schwab.** 1977. Processing of streptococcal cell walls by rat macrophages and human monocytes in vitro. Infect. Immun. **17**:591–598.
43. **Spitznagel, J. K., K. J. Goodrum, and D. J. Warejcka.** 1983. Rat arthritis due to whole group B streptococci. Clinical and histopathologic features compared with groups A and D. Am. J. Pathol. **112**:37–47.
44. **Watanabe, T., H. Shimohashi, Y. Kawai, and M. Mutai.** 1981. Studies of streptococci. I. Distribution of fecal streptococci in man. Microbiol. Immunol. **25**:257–269.
45. **Webster, G. F., U. R. Nilsson, and W. P. McArthur.** 1981. Activation of the alternative pathway of complement in human serum by *Propionibacterium acnes* (*Corynebacterium parvum*) cell fractions. Inflammation **5**:165–176.
46. **Weiss, N.** 1981. Cell wall structure of anaerobic cocci. Rev. Inst. Pasteur Lyon **14**:53–59.
47. **Wensinck, F., and J. P. van de Merwe.** 1981. Serum agglutinins to *Eubacterium* and *Peptostreptococcus* species in Crohn's and other diseases. J. Hyg. **87**:13–24.