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# Arthropathic Properties of Gonococcal Peptidoglycan Fragments: Implications for the Pathogenesis of Disseminated Gonococcal Disease

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**We examined the arthropathic activity of purified peptidoglycan (PG) fragments derived from (i) lysozyme-resistant, extensively *O*-acetylated PG from *Neisseria gonorrhoeae* FA19 (*O*-PG), and (ii) lysozyme-sensitive, *O*-acetyl-deficient PG from *N. gonorrhoeae* RD5 (non-*O*-PG). Male Lewis rats were injected intradermally in the tail with 200 μg of PG emulsified in mineral oil and water (1:1) or with the oil and water emulsion alone (controls). Quantitation of hind paw size indicated that macromolecular PG of various chemical and physical forms induced paw swelling (*P* versus controls, less than 0.01) that was evident at about day 14 and that reached a maximum at about day 24. PG-mediated paw swelling was accompanied by intense synovitis with some cartilage and bone involvement. The minimal arthropathic dose of soluble macromolecular PG was 20 μg per rat. Of particular interest was that macromolecular *O*-PGs from strain FA19 caused considerably more extensive swelling than did either their RD5 non-*O*-PG counterparts or the homologous FA19 PG that had been de-*O*-acetylated by mild alkali treatment. This suggested that the persistence of hydrolase-resistant high-molecular-weight fragments, afforded by extensive *O*-acetylation, may be important for optimal expression of arthropathic activity. However, oligomeric PG was not an absolute requirement, since even low-molecular-weight fragments, including the anhydro-muramyl-containing disaccharide peptide monomer released by growing gonococci, were also arthritogenic. Experiments employing purified gonococcal lipopolysaccharide indicated that the arthropathic activity of PG preparations was not due to contaminating lipopolysaccharide. Based on the arthritogenicity of gonococcal PG in this model system, we suggest that PG may play a role in the pathogenesis of gonococcal arthritis, and that such an activity might be potentiated by the persistence of hydrolase-resistant *O*-PG.**

We are testing the hypothesis that gonococcal peptidoglycan (PG) fragments, released by bacterial (25, 32, 33) and host PG hydrolases (27), contribute to the intense inflammatory reactions characteristic of gonococcal disease. Such conditions include disseminated gonococcal infection, the clinical hallmark of which is an arthritis syndrome in which viable bacteria are only infrequently demonstrated in affected joints (10) and in which immunological injury likely contributes to tissue damage, at least in some clinical stages of disease (10, 36).

As part of the test of this hypothesis, our objectives have been (i) to compile an inventory of purified PG fragments that likely gain access to host tissues in vivo, and (ii) to test their ability to initiate biological reactions associated with modulation of immune and inflammatory responses. In regard to the first objective, our previous data (25, 27, 28, 32, 33) argue that the release of PG fragments of widely diverse sizes and chemical forms might be realistically achievable during natural infection. These fragments range from the anhydro-muramyl-containing disaccharide peptide monomers (ca. 950 daltons), released by growing gonococci as a result of PG turnover (25, 32, 33), to enormous soluble fragments (greater than $10^6$ daltons) released by the action of human PG-degrading enzymes present in polymorphonuclear leukocytes and serum (27). Furthermore, because the vast majority of gonococcal isolates possess PG that resists complete degradation by commercial and human PG hydrolases (a property directly related to the presence of numerous *O*-acetyl substituents on the glycan backbone; 2, 26–28, 35), it appears that these high-molecular-weight fragments might have an exceptional capacity to persist in the oligomeric form.

In regard to the second objective, the essential conclusion is that purified PG fragments collectively do initiate biological reactions compatible with their playing a role in modulation of the host reaction to gonococcal infection. Gonococcal PG-mediated activities include (i) intrinsic toxicity for human fallopian tube mucosa (21), (ii) consumption of human complement and generation of active mediators of inflammation (24), and (iii) modulation of the blastogenic response of human and murine lymphocytes (R. A. Bennett, R. S. Rosenthal, and C. E. Wilde III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, O40, p. 49). Furthermore, recent studies (J. M. Krueger, R. S. Rosenthal, S. A. Martin, J. Walter, D. Davenne, S. Shoham, S. L. Kubillus, and K. Biemann, manuscript in preparation) have indicated that anhydro-muramic acid-containing monomers, released by growing gonococci, induce slow-wave sleep in experimental animals. This conclusion is consistent with the original observations on the somnogenic properties of muramyl peptides (18) in that a major component of anhydro-monomers (the disaccharide tetrapeptide) is identical to the naturally occurring somnogenic factor (factor S) present in numerous animals including humans (17, 20, 32). The structural requirements of these diverse activities have not been completely defined. However, the complement-consuming and the blastogenic activities appear to depend on high-molecular-weight, glycosidically linked oligomers for optimal activity. Similar structural features are apparently important for many of the biological activities mediated by PG

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from gram-positive bacteria, e.g., complement activation (12, 37), pyrogenicity (22), and arthritogenicity (4, 15).

Current experiments posed a rather direct test of our hypothesis and assessed the biological effects of gonococcal PG in vivo by examining the arthropathic properties of purified PG fragments in a rat model. We have found that gonococcal PG of various chemical and physical forms does induce arthritis in rats, and that this activity is potentiated by hydrolase-resistant, extensively *O*-acetylated PG.

(A preliminary report of some of these experiments has appeared previously [R. S. Rosenthal and T. J. Fleming. 1985. Arthropathic properties of gonococcal peptidoglycan, p. 352–359. *In* G. Schoolnik, ed., *The Pathogenic Neisseriae*. American Society for Microbiology, Washington, D.C.].)

## MATERIALS AND METHODS

**Preparation and chemical properties of purified gonococcal PG fragments.** Intact (insoluble) PG, the starting material for preparation of various PG fragments, was purified from exponential-phase, nonpiliated, transparent clones of *Neisseria gonorrhoeae* strains FA19 (provided by P. F. Sparling, Chapel Hill, N.C.) and RD5 (provided by F. E. Young, Rochester, N.Y.) as previously described (27, 29). Strain FA19 was the source of extensively *O*-acetylated PG (*O*-PG); approximately one-half of the disaccharide peptide monomer subunits of FA19 PG are substituted with *O*-acetyl groups (1, 5, 26, 35), probably located on the number 6 carbon of the muramic acid moiety (23). Strain RD5 was the source of *O*-acetyl-deficient PG (non-*O*-PG). Previous reports have indicated that less than 14% of the monomer subunits of RD5 PG are *O*-acetylated (26, 35). In fact, more recent experiments (S. Martin, K. Biemann, and R. Rosenthal, unpublished observations) with the powerful techniques of fast-atom bombardment mass spectrometry and reverse-phase high-performance liquid chromatography applied to underivatized gonococcal PG samples suggested that PG monomers from RD5 may be devoid of *O*-acetyl substituents. To remove *O*-acetyl groups selectively, purified FA19 *O*-PG was treated with 0.01 N NaOH for 10 h at 25°C. This mild alkali treatment reduced the extent of *O*-acetylation of *O*-PG to that of non-*O*-PG from strain RD5 and, as reported previously (28), neither promoted the beta elimination of lactyl peptides nor otherwise caused detectable changes in PG composition or structure.

The following classes of purified PG fragments, derived from the appropriate intact PG, were used in arthritis studies.

(i) Sonicated (S) PG, e.g., S-*O*-PG and S-non-*O*-PG, prepared from *O*-PG and non-*O*-PG as described previously (24), were heterogeneous mixtures of soluble fragments (greater than $10^6$ daltons) that simulated the large-molecular-weight fragments released by the action of host PG hydrolases (27), e.g., human polymorphonuclear leukocyte lysozyme. S-PG preparations used in experiments contained between less than 0.1 to 0.9% (wt/wt) non-PG amino acids, and between 0.05 and 0.25 ng of lipopolysaccharide (LPS) per μg of PG, as determined by the *Limulus* amoebocyte lysate assay (see below). S-PGs possess the native extent of peptide cross-linking (approximately 40%; 29).

(ii) Chalaropsis PG fragments, derived from non-*O*-PG by complete digestion with Chalaropsis B muramidase as described previously (21, 29), were chemically defined mixtures of reducing, un-cross-linked disaccharide peptide monomers (29%), peptide-cross-linked dimers (40%), trimers (22%), and tetramers (9%). The Chalaropsis B enzyme breaks appropriate glycosidic linkages in PG independent of the presence or absence of *O*-acetyl substituents (2, 26). Chalaropsis PG fragments used in experiments contained no detectable non-PG amino acids at a sensitivity that should have easily detected less than 0.1% (wt/wt) and no more than 0.012 ng of LPS per μg of PG.

(iii) The anhydro monomer, derived from non-*O*-PG as described previously (21), was a chemically defined mixture of the nonreducing anhydro-muramyl-containing monomers released by growing gonococci. The anhydro monomer used in experiments contained virtually no non-PG amino acids and no more than 0.012 ng of LPS per μg of PG. The major components of this preparation were previously identified as *N*-acetylglucosaminyl-β-1→4-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid and the corresponding disaccharide tetrapeptide containing a C-terminal D-alanine (32). Very recent experiments (S. Martin, K. Biemann, and R. Rosenthal, unpublished observations) employing fast-atom bombardment mass spectrometry proved the structure of the two principal fragments of anhydro monomer, demonstrated several previously undetected minor PG components, and detected no non-PG contaminants. Structural formulae for the major components of Chalaropsis and anhydro monomers derived from gonococcal PG have appeared previously (32).

**Arthritis model.** Groups of eight specific-pathogen-free, male Lewis rats (200 to 225 g; Harlan Sprague Dawley, Inc., Walkersville, Md.) were injected intradermally in the tail with 200 μl of test substances emulsified in an equal volume mixture of oil (Freund incomplete adjuvant; Difco Laboratories, Detroit, Mich.) and pyrogen-free water or with the oil-water emulsion alone (controls). In some experiments, test substances were administered in aqueous solution by intraperitoneal or intravenous injection. The usual dose of PG was 200 μg per rat; in all experiments reported here, each rat received only a single injection of PG. In some experiments, the arthropathic activity of gonococcal LPS purified from T1 clones of strain 2686 (a gift from M. A. Melly, Nashville, Tenn.) and muramyl dipeptide (G.I.R.P.I., Paris) was also tested in this model system. At intervals after injection, hind paw size was measured in planes parallel and perpendicular to the plane of the lateral malleoli with a dial thickness gauge and expressed, in millimeters, as the mean score for each group. The net increase in the area under the curve of joint score versus time was estimated for each rat by using the trapezoidal rule, and the mean areas between groups in a given experiment were compared with a two-sample *t* test.

**Analytical methods.** Limulus amoebocyte lysate tube assays for quantitation of LPS were performed according to the manufacturer's instructions (Associates of Cape Cod, Inc., Woods Hole, Mass.) with gonococcal LPS purified from strain 2686 as a standard. Previously described procedures were employed for analysis of the amino acid content of PG preparations (29) and for determination of the percent *O*-acetylation of PG, i.e., the percentage of monomer subunits substituted with *O*-acetyl groups (35). Additional analytical procedures used for quality control of PG preparations were as described previously (21, 29, 32).

## RESULTS

**Arthropathic activity of macromolecular gonococcal PG.** Intradermal injections of extensively *O*-acetylated macromolecular PG, i.e., intact *O*-PG and S-*O*-PG (200 μg per rat), consistently induced extensive hind paw swelling (*P* versus

control, less than 0.01), which was first evident about 14 to 17 days after injection and maximal between days 24 and 28 (Fig. 1). As indicated in Fig. 2, 40 μg, but not 5 μg, of S-*O*-PG caused significant hind paw swelling; in other experiments (data not shown) the minimal arthropathic dose of S-*O*-PG was found to be 20 μg per rat. S-*O*-PG-treated rats underwent rapid, but only partial, recovery after the initial period of peak joint scores. In fact, considerable paw inflammation persisted for at least 100 days, and some animals even exhibited a secondary increase in joint score that approached initial peak levels (Fig. 3). Radiological (Fig. 4) and histological (Fig. 5) studies indicated that PG-mediated paw swelling (Fig. 6A) was accompanied by intense synovitis with some involvement of cartilage and bone. Whereas PG frequently induced front paw swelling, this parameter was not as sensitive or as reliable an indicator of arthritis as was hind paw swelling. Arthritic rats also tended to exhibit other gross evidence of inflammatory damage, e.g., extensive inflammation of the external genitalia (Fig. 6B) and tail necrosis distal to the site of injection. Preliminary results of gross and histological examination of internal viscera were unremarkable.

In contrast to S-*O*-PG, which consistently caused severe arthritis, corresponding non-*O*-PG preparations caused severe disease on a sporadic basis only and, overall, tended to induce relatively mild and infrequent disease. In three of four experiments performed as in Fig. 1, the mean joint score of non-*O*-PG-treated animals, although greater than that of controls (*P* less than 0.01), was markedly less than the joint score of *O*-PG-treated animals (*P* varied from about 0.05 to less than 0.01). Thus, these data (Fig. 1) suggested that extensive *O*-acetylation might be required for maximal arthropathic activity of gonococcal PG. To confirm that the differences in arthropathic activity between FA19 S-*O*-PG and RD5 non-*O*-PG (Fig. 1) were related specifically to *O*-acetylation and not to some unknown structural differences in the PGs of these heterologous strains, we compared the activities of S-*O*-PG from FA19 and of the homologous PG that had been treated with mild alkali to cause the complete and specific removal of *O*-acetyl substituents. Indeed, de-*O*-acetylated PG caused greatly reduced paw inflammation as compared with the corresponding extensively *O*-acetylated PG (Fig. 2), thus confirming the importance of *O*-acetylation in this activity.

The effect of the route of administration on PG-mediated paw swelling in this model system was also tested. Macromolecular PGs were not arthritogenic when injected in aqueous vehicles, either intraperitoneally (data not shown) or intravenously (Fig. 7). Thus, to date, the arthropathic potential of gonococcal PG has been achieved only when the PG has been injected in an oil-water emulsion.

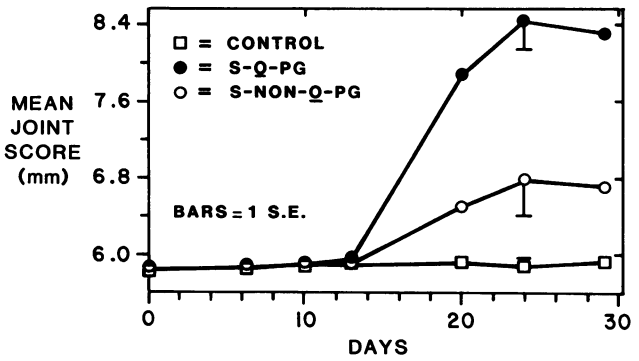


FIG. 1. Induction of hind paw swelling in male Lewis rats after intradermal injection in the tail of purified macromolecular gonococcal PG (200 μg).

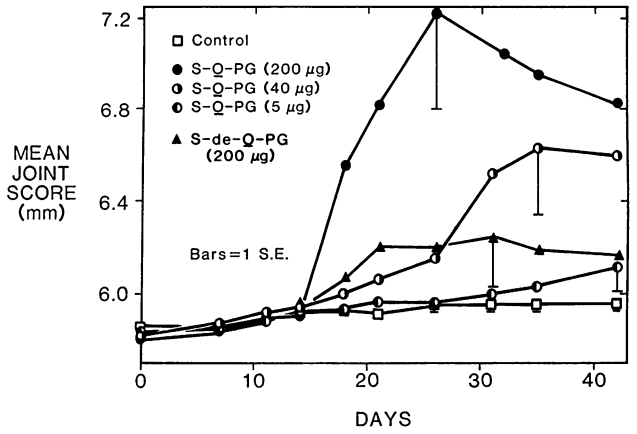


FIG. 2. Dose dependency of S-*O*-PG-mediated arthritis and effect of de-*O*-acetylation on the arthropathic activity of S-*O*-PG. De-*O*-acetylated PG (de-*O*-PG) was obtained by treating FA19 S-*O*-PG with mild alkali, which resulted in the complete and specific removal of *O*-acetyl substituents.

**Arthropathic activity of low-molecular-weight PG fragments.** Although hydrolase-resistant oligomeric PG was apparently important for optimal expression of arthropathic activity (Fig. 1 and 2), high-molecular-weight PG was found not to be an absolute requirement. Thus, even the low-molecular-weight fragments produced by complete digestion of PG by Chalaropsis muramidase retained arthropathic activity (*P* versus controls, less than 0.01; Fig. 7), although the biological response seemed delayed and usually less intense than that caused by macromolecular *O*-PG. Furthermore, the class of purified, anhydro-muramyl-containing disaccharide peptide monomers (anhydro monomer), the major PG fragments released by growing gonococci, was also arthritogenic (*P* less than 0.05; Fig. 3). Commercially available muramyl dipeptide appeared somewhat less active than the natural gonococcal PG fragments (Fig. 3) in this model system, but these differences were not statistically significant. It should be pointed out that additional column controls (21), prepared to control for biologically active contaminants that might have been acquired during the purification of low-molecular-weight gonococcal PG fragments, were completely inactive.

**Failure of gonococcal LPS to induce arthritis in this model system.** Routine chemical procedures as well as mass spectrometry have indicated that biologically active PG preparations contain very little (less than 1%) detectable chemical contaminants. However, such techniques would not necessarily detect low levels of gonococcal LPS that, conceivably, might be biologically active. Indeed, it has been reported (11) that 5 μg or greater of gonococcal LPS does induce acute and chronic synovitis in rabbit knees. In those experiments (11), however, the LPS was injected directly into the knee, and thus there is no precedence for the arthritogenicity of gonococcal LPS after injection at remote sites, such as we have employed. At any rate, to examine the possibility that LPS was the active factor in PG preparations, we (i) measured the amount of LPS in PG preparations by the *Limulus* amoebocyte lysate assay and (ii) tested the arthropathic

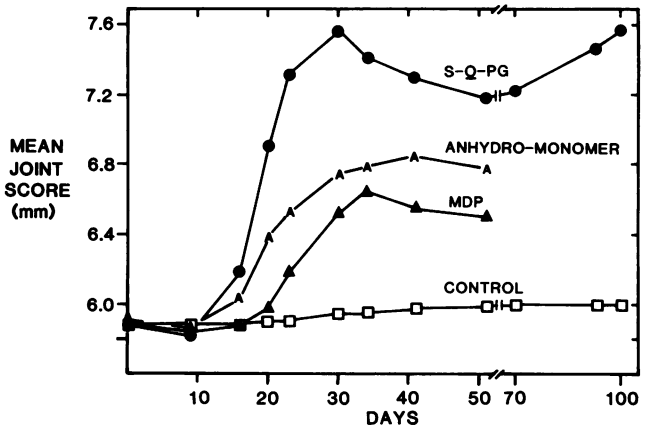


FIG. 3. Arthropathic activity of purified anhydro monomer, muramyl dipeptide (MDP), and S-*O*-PG (positive control), each administered at 200 μg per rat.

activity of purified gonococcal LPS in amounts that were in great excess of the amount present in PG samples. LPS in doses of 0.01, 1, 5, and 10 μg caused no significant paw swelling when injected intradermally, although 5 and 10 μg did cause detectable swelling in a small percentage of animals. In each case, positive controls (S-PG or Chalaropsis PG fragments) induced significant paw swelling, as before. Thus, gonococcal LPS, in levels that were as much as 2,000 times the maximal amount present in arthritogenic doses of 20 μg of S-PG and greater than 4,000 times that present in arthritogenic doses of Chalaropsis PG fragments and anhydro monomer, was not active in this model system. We conclude that the arthropathic activity of gonococcal PG fragments is not due to contaminating LPS.

## DISCUSSION

These studies have demonstrated that purified gonococcal PG fragments, similar to those that might gain access to host tissues during natural infection, possess arthropathic activity in a rat model. Soluble macromolecular PG, e.g., S-*O*-PG

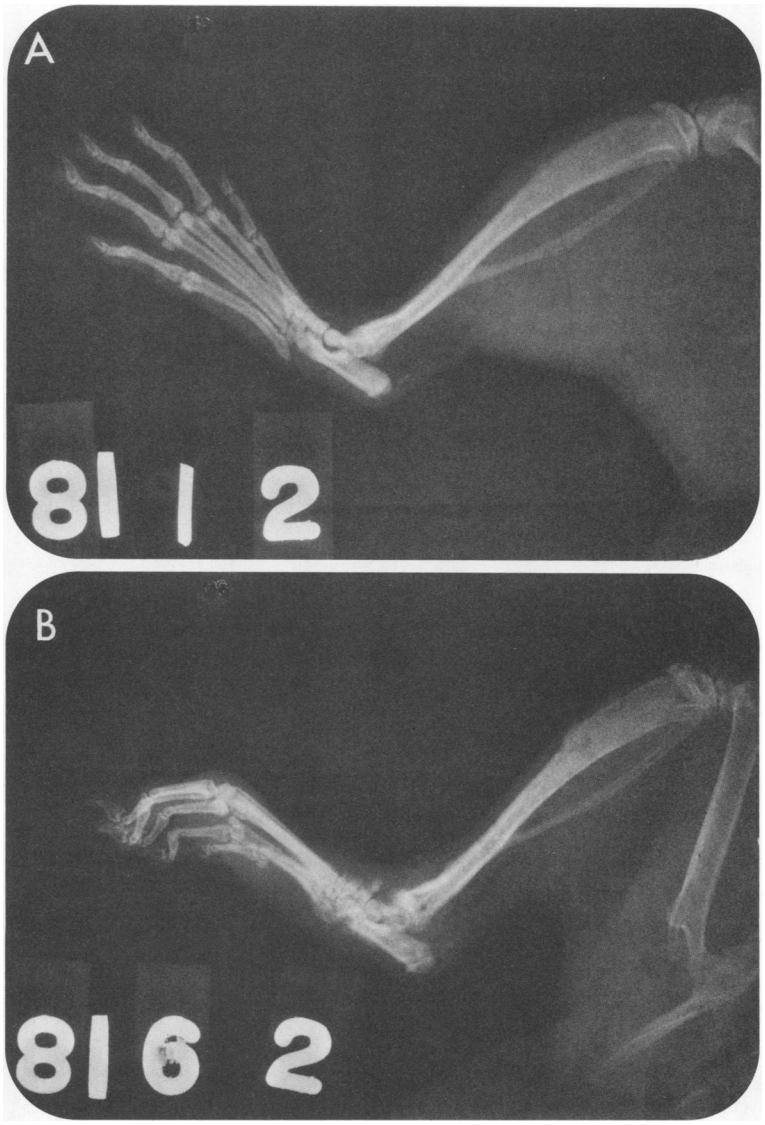


FIG. 4. X-ray of typical control (A) and S-*O*-PG-treated (B) rats on day 32.

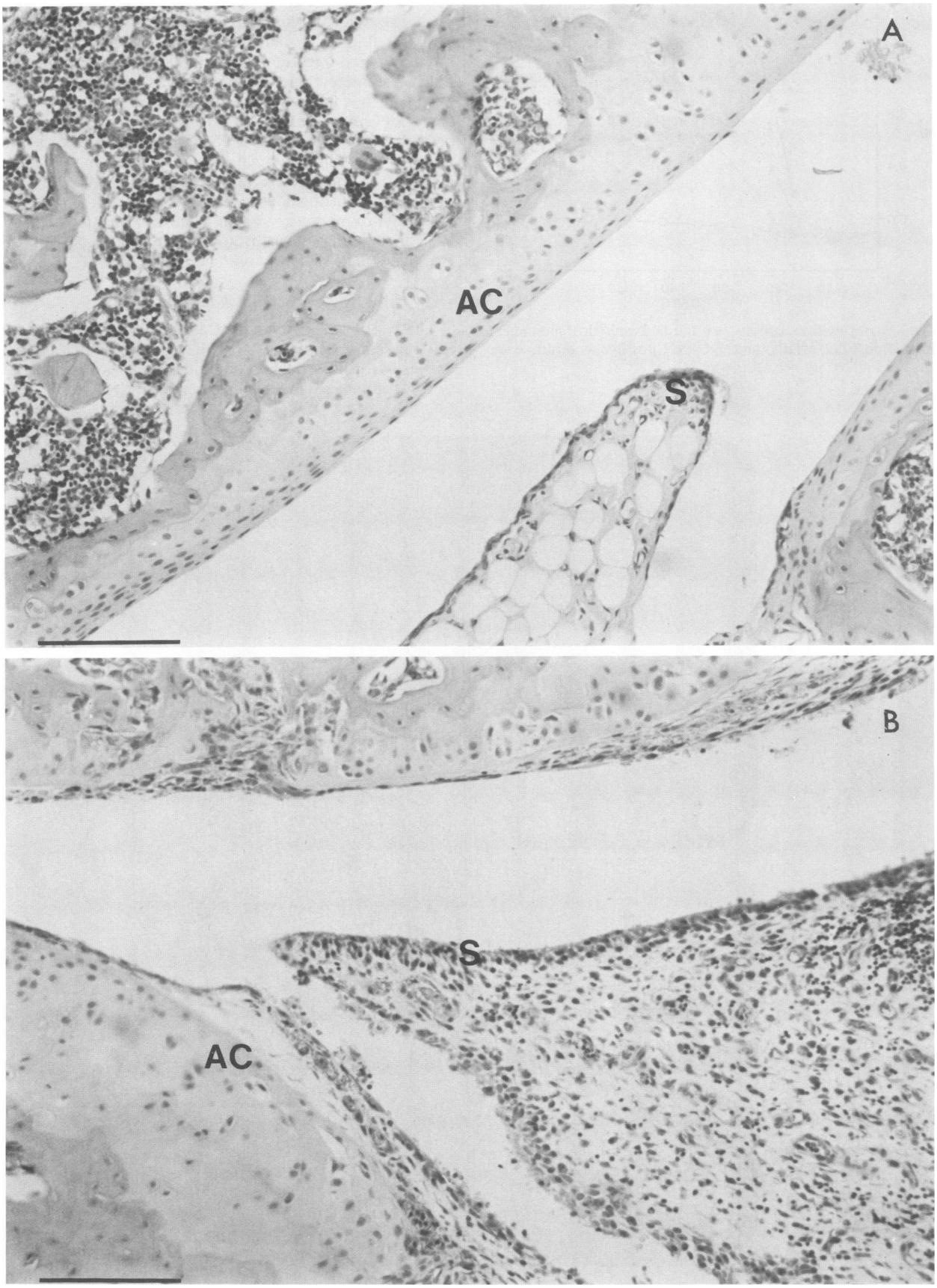


FIG. 5. Histology of joint tissue obtained from rats at 42 days. (A) Section of control ankle with no evidence of inflammation. (B) Section of ankle of S-*O*-PG-treated rat showing intense inflammatory changes in synovium (S) and articular cartilage (AC). Hematoxylin and eosin stain. Bars, 50 μm.

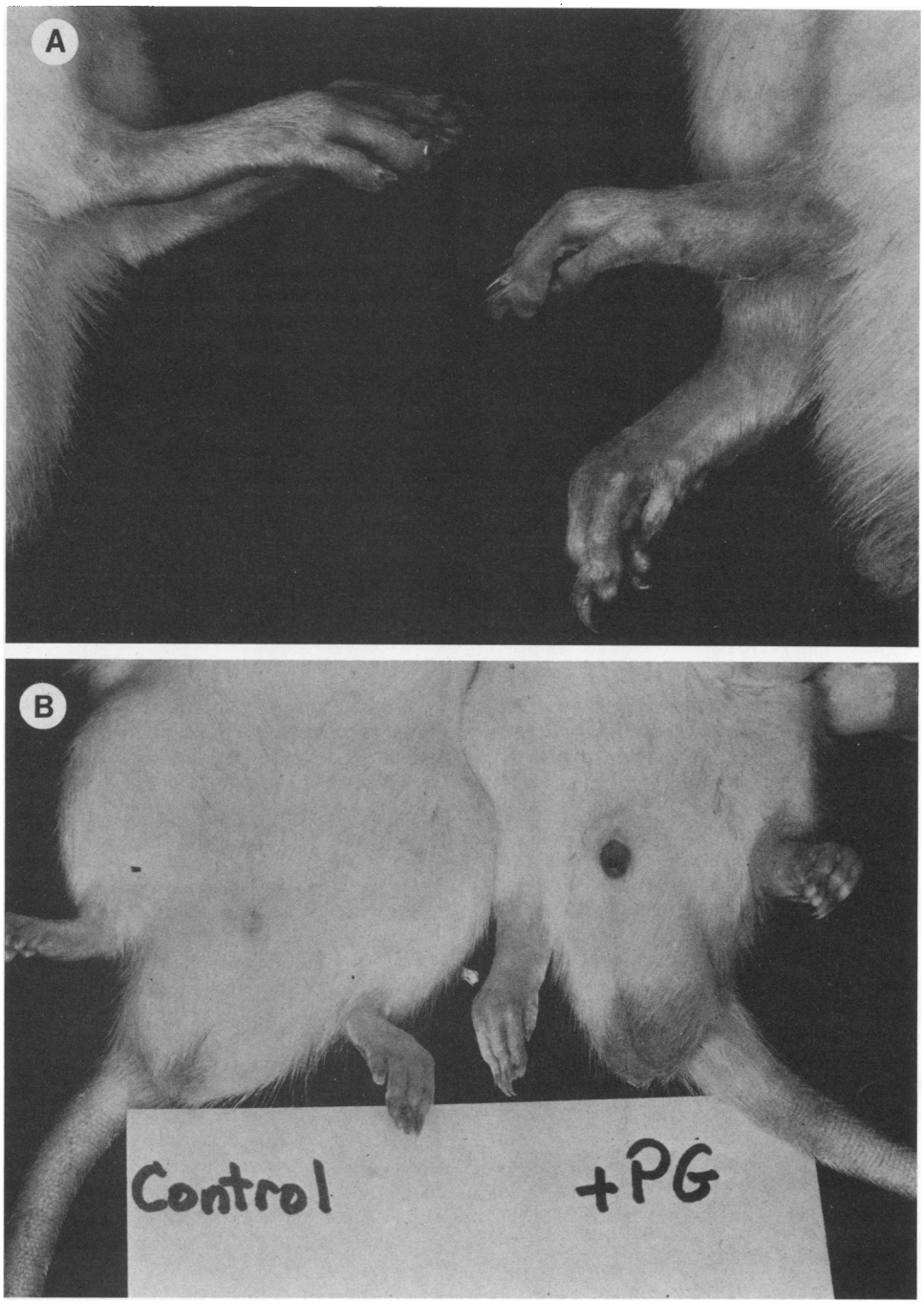


FIG. 6. Gross pathology of PG-treated rat (right) 32 days after intradermal administration of 200 μg of S-*O*-PG demonstrating hind paw swelling (A) and inflammation of external genitalia (B).

and S-non-*O*-PG, and various classes of low-molecular-weight PG fragments, e.g., anhydro monomer, both induced significant paw swelling. However, macromolecular *O*-PG alone was able to evoke the maximal arthritic response observed in these experiments. Because *O*-PG is quite resistant to PG-degrading enzymes, compared with PG that lacks extensive *O*-acetylation (27, 28), we currently favor the hypothesis that the enhanced arthritogenicity of gonococcal *O*-PG reflects its ability to persist as PG oligomers in vivo. Indeed, the work of Schwab, Ginsburg, and their respective co-workers (4, 6, 7, 30, 34) has provided convincing precedent that PG hydrolase resistance, and the attending persistence of PG in tissues, is a key factor in the induction of arthritis by cell walls derived from group A streptococci and other gram-positive bacteria. The structural basis for the resistance of streptococcal PG, however, related to its covalent attachment to the group-specific polysaccharide and to numerous unsubstituted amino groups (8, 30) rather than to the presence of *O*-acetylated glycan chains. Nevertheless, our results on the enhanced arthritogenicity of hydrolase-resistant *O*-PG are consistent with the argument that PG persistence may be a common denominator for cell

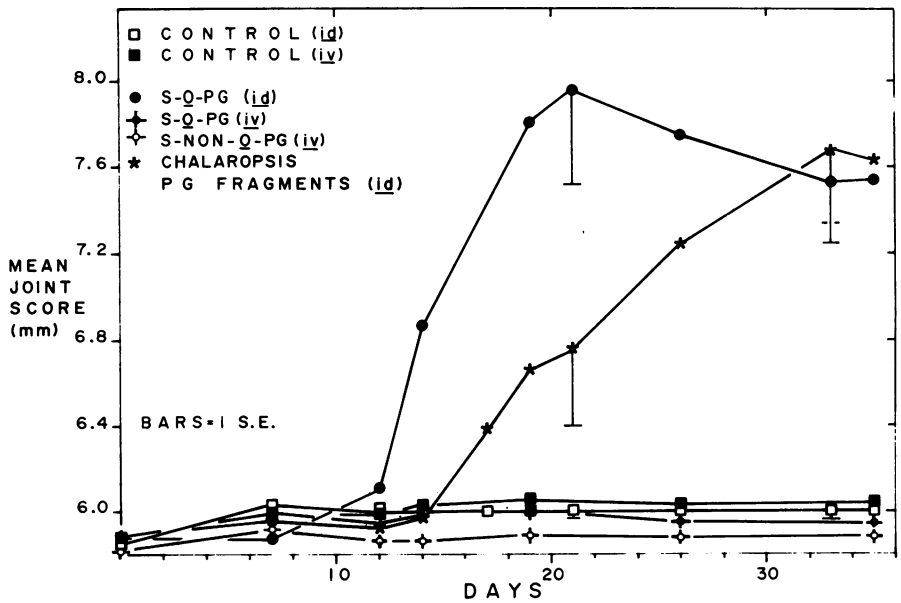


FIG. 7. Effect of route of administration on arthropathic activity of macromolecular PG (S-*O*-PG and S-non-*O*-PG) and induction of arthritis by low-molecular-weight fragments (Chalaropsis PG fragments) obtained by complete digestion of non-*O*-PG by Chalaropsis B muramidase. Each PG was administered at a dose of 200 μg per rat.

wall-induced arthritis. These data may also offer an explanation why select gram-negative bacteria are closely associated with infections that lead to "sterile" arthritic joints (10). Thus, it would be interesting to survey some of these causative agents of "reactive" arthritis, e.g., *Yersinia enterocolitica*, for the presence of hydrolase-resistant *O*-acetylated PG that might gain access to host tissues. To date, the presence of *O*-acetylated PG seems well established only in *Proteus* species (9, 19) and staphylococci (3, 13, 14) in addition to gonococci (2, 5, 35).

Although macromolecular gonococcal PG may be required for the maximal expression of arthropathic activity observed in these experiments, it was of interest that even the low-molecular-weight anhydro monomers retained significant arthropathic activity. Indeed, this result is of great potential relevance since the class of anhydro monomers represents the principal PG fragments released by growing gonococci as a result of PG turnover (25, 32, 33).

One of the main issues of these initial studies was to assess the probability that the arthritogenicity of PG preparations might actually be due to the presence of arthropathic non-PG contaminants. Of course, in complex biological systems (such as the rat arthritis model), which employ test substances purified from intact bacteria, it is difficult to prove specific cause-and-effect relationships beyond a shadow of a doubt. In theory, there could always be some undetected contaminant that is responsible for the activity and that copurifies with the putative effector molecules. Yet, the data seem to argue convincingly that the arthritic response to PG preparations can be accounted for solely by the presence of the PG itself. First, several analytical procedures failed to detect non-PG contaminants in some biologically active PG preparations at sensitivities that should have easily detected contaminants at the level of 0.1 to 1% (wt/wt). Thus, by the most conservative estimate, and taking 20 μg as the minimal active dose of S-*O*-PG, any undetected arthropathic contaminant would have to be active at a dose of less than 0.2 μg per rat to confound our essential interpretation that PG is the active arthritogen.

Second, gonococcal LPS (the most likely trace contaminant that, conceivably, could be active in this model system) had little or no activity in amounts up to 10 μg per rat; this represents greater than 4,000 times the amount of LPS present in biologically active doses of 200 μg of low-molecular-weight PG fragments and at least 2,000 times the amount of LPS in the minimal active dose of 20 μg of S-*O*-PG. The relative inability of a gonococcal LPS to cause arthritis in this rat model is, itself, a notable side issue, in light of the evidence of Goldenberg et al. that gonococcal LPS (not unlike LPS from other bacteria) induces arthritis in rabbits (11). There are numerous technical differences which could explain the differing results. One of the most likely, however, relates to route of administration since these investigators (11) injected LPS directly into the knee joints. In contrast, we felt that a positive test for arthropathic activity should require that test substances exert their activity after injection at a remote site, rather than simply induce local inflammation after intraarticular injection. Accordingly, we settled upon intradermal administration of test substances. At present, we cannot explain the apparent requirement that the PG be injected in an oil-water vehicle. However, there is some precedence that the "depot" effect of certain oils is necessary for some soluble PG fragments, chemically distinct from gonococcal PG fragments, to exert their arthropathic potential (16).

In summary, the ability of purified gonococcal PG to induce arthritis in this animal model is consistent with the hypothesis that PG plays a role in the pathogenesis of gonococcal arthritis.

## ACKNOWLEDGMENTS

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