American Journal of Pathology, Vol. 132, No. 2, August 1988

Mast Cell Activation by Group A Streptococcal Polysaccharide in the Rat and Its Role in Experimental Arthritis

FREDERIC G. DALLDORF, MD, SONYA K. ANDERLE, MS, ROGER R. BROWN, BSc, and JOHN H. SCHWAB, PhD

From the Departments of Pathology and Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina

Acute edematous responses were induced in Sprague-Dawley rats by the intravenous injection of group-specific polysaccharide (PS) isolated from group A streptococci. Thirty minutes after the intravenous injection of PS there was marked degranulation of subcutaneous and periarticular mast cells in all 4 feet, carbon particle labeling of adjacent venules, and an 8-fold increase in Evans blue dye content of the extremities. This acute reaction to PS was completely blocked by pretreatment with compound 48/80, but the polyarticular relapsing arthritis following the systemic injection of an arthropathic dose of streptococcal cell wall fragments containing large, covalently bound peptidoglycan-polysaccharide (PG-PS) was not blocked. (Am J Pathol 1988, 132:258–264)

INTRAPERITONEAL INJECTION of Sprague-Dawley rats with a sterile aqueous suspension of peptidoglycan-polysaccharide fragments (PG-PS) from Group A streptococci induces a prolonged inflammatory polyarthritis.[1] Most of the PG-PS accumulates in the liver, spleen, and lymph nodes, where it causes acute inflammation but only moderate chronic disease in this outbred strain of rat. A small amount of the material accumulates about the joints of all 4 extremities, where it elicits an acute diffuse polyarticular arthritis that starts as a focal vasculitis and synovitis 16 hours after injection, peaks 3 days later, and then recedes. This is followed by chronic relapsing polyarticular arthritis that leads to pannus formation, destruction of articular cartilage, and ankylosis.[1,2] The localization of the PG-PS within the reticuloendothelium of the rat is understandable as the material quickly gains access to circulating leucocytes and plasma via the peritoneal lymphatics, but the mechanism for its selective deposition in and about the joints is still unknown. Morphologic studies demonstrated early localization of cell wall material within neutrophiles passing through venule walls in the synovium 16 hours into the reaction but failed to explain this selective vascular response.[2] Recently Chetty, Brown, and Schwab[3] reported that purified group-specific polysaccharide (PS) isolated from group A streptococcal cell wall fragments, when injected intravenously, causes transient acute edema largely confined to the limbs. Because the PG-PS preparations contain some free PS together with the cell wall fragments it was suggested that an acute vascular reaction to small amounts of PS might predispose the extremities to the deposition of PG-PS and the later development of arthritis.[3] This concept was further supported by the observation that more purified preparations of PG-PS produced little arthritis in Sprague-Dawley rats unless PS was added to the intraperitoneal innoculum.[3] The purpose of this study is to investigate the pathogenesis of the edema-producing properties of PS in the rat and

Supported by research grant AM 25733 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

Accepted for publication March 31, 1988.

Address reprint requests to Frederic G. Dalldorf, MD, Department of Pathology, School of Medicine, CB #7525, University of North Carolina, Chapel Hill, NC 27514.

to evaluate its possible role in PG-PS induced arthritis.

Materials and Methods

Preparation of Peptidoglycan-Polysaccharide Fragments and Group-Specific Cell Wall Polysaccharide

Group A, type 3, strain D-58 streptococci were grown in Todd-Hewitt broth, harvested, washed, and disrupted in a Braun MSK shaker (Braun Instruments, San Francisco, CA) and the cell wall fragments purified by differential centrifugation and enzyme treatment, as described previously.[4] The group-specific polysaccharide was isolated from the cell walls by the method of Krause[5] as modified by Chetty, Klapper, and Schwab.[4] A cell wall preparation was suspended in formamide (5 mg/ml) and extraction was performed at 150 C for 20 minutes. After being cooled overnight at 4 C, the extracted preparation was centrifuged (36,000*g*, 30 minutes, 4 C), the supernatant was mixed with 2 volumes of acid-alcohol (95% ethanol and 5% 1 N HCl), and the mixture was centrifuged (12,000*g*, 30 minutes, 4 C). The PS in the alcoholic supernatant was precipitated by adding 5 volumes of acid-acetone (99% acetone and 1% HCl) and a few crystals of sodium acetate to the supernatant. This was held at 4 C for 72 hours. The acid-acetone precipitable carbohydrate was dissolved in distilled water, clarified by centrifugation, and treated with acid-alcohol and acid-acetone once more. The PS obtained from the second acid-acetone treatment was suspended in distilled water, dialyzed against distilled water for 48 hours at 4 C, and passed through Dowex-50 × 8 (200-400 mech) cation exchange resin (Bio-Rad Laboratories, Richmond, CA) before lyophilization. The lyophilized PS was suspended in an appropriate amount of distilled water and was treated again with acid-alcohol and acid-acetone before use. The rhamnose concentration was determined on each batch by the method of Dische and Shettles.[6]

Animals

Outbred female Sprague-Dawley rats were purchased from Zivic-Miller Laboratories, Allison Park, PA, and weighed 120–160 gms at the beginning of the experiments.

Histologic Methods

Rats were killed by ether overdose and complete autopsies performed. Tissues were fixed in 10% phosphate buffered formalin and in selective experiments sections prepared from the skin, snout, ears, tail, lungs, gut, liver, spleen, mediastinal lymph nodes, thymus, kidneys, and the left front and rear feet. The feet were decalcified with formic acid sodium citrate solution and sectioned longitudinally with the skin. All histologic sections were stained with toluidine blue and some with hematoxylin and eosin (H&E) and periodic acid-Schiff digest.

Morphologic Studies

Fourteen rats were injected intravenously with 6 μg PS/g body weight in PBS. Seven of these were sacrificed after 30 minutes and 7 after 6 hours. Three of the 30-minute rats were also injected intravenously with 1 ml of a 5% suspension of India ink (Higgins Drawing Ink) in PBS 25 minutes before sacrifice. Two additional control rats received PBS intravenously 30 minutes before sacrifice and 1 of these was also injected with India ink 25 minutes before sacrifice. Three of the 7 rats held 6 hours after PS injection were also injected with India ink 25 minutes before sacrifice. Two additional control rats were injected with PBS and sacrificed after 6 hours. One of these controls was also injected with India ink 25 minutes before sacrifice. All but 1 of the 14 rats receiving PS developed gross edema of all 4 feet. The edema had subsided completely in the rats held 6 hours. None of the 4 control rats injected with PBS developed edema.

Measurement of Permeability Response

The method of Udaka, Takeuchi, and Movat[7] was used. Each rat received 6.5 mg filtered Evans blue in PBS intravenously 45 minutes before sacrifice. The right hind and fore feet were removed 2 mm above the ankle from each rat, frozen in liquid nitrogen, pulverized and the dye extracted with PBS. The optical density was measured in a spectrophotometer at 620 mu and the quantity of dye per combined sample calculated using a standard dye dilution curve.

Compound 48/80 Degranulation of Mast Cells

The method used was a modification of that of Riley and West.[8] Treated rats received increasing intraperitoneal doses of 48/80 for 5 days. On the first day each rat received 100 μg intraperineally in the afternoon. On the second day they received 200 μg twice, once in the morning and once in the afternoon, on the third day 300 μg twice, and on the fourth day 400 μg twice. The fifth day the rats were injected once

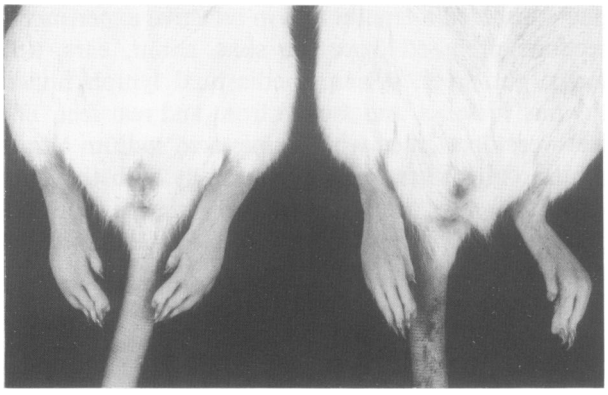

Figure 1—PS-induced edema. The rat on the left was injected intravenously with 6 μg PS/g body weight 30 minutes before they were killed. The rat on the right was the PBS control. Note marked foot swelling on the left.

in the morning with 500 μg and experiments were performed on the morning of the sixth day. All rats became short of breath and had edema of their feet and nose following each injection. Using this modified schedule none of the rats died and they all gained weight, as did controls. Control rats were injected twice a day with a comparable volume of PBS.

Compound 48/80 Inhibition of PS-Induced Edema

Twenty rats were divided into 3 groups. In Group I, 6 rats were injected intraperineally with graded doses of 48/80 for 5 days. On day 6 they were injected IV with 6.5 mg Evans blue in PBS 45 minutes before sacrifice and PBS alone intravenously 30 minutes before sacrifice. In Group II, 7 rats were injected intraperineally with graded doses of 48/80 for 5 days. On day 6 they were injected intravenously with 6.5 mgm Evans blue in PBS 45 minutes before sacrifice and 6 μg PS/g body weight 30 minutes before sacrifice. In Group III, 7 rats were injected intraperineally for 4 days with PBS. On day 6 they were injected first with Evans blue in PBS 45 minutes before sacrifice and then 6 μg PS/g body weight PS 30 minutes before sacrifice. All rats were injected intravenously through the tail veins under light ether anesthesia and were killed with ether. Observations were made of the gross appearance of all rats. The left front and rear feet were prepared for histology and sections stained with toluidine blue. The right feet were excised 2 mm proximal to the wrist or ankle and analysed for total content of Evans blue dye.

Compound 48/80 and Experimental Arthritis

Thirty-four rats were divided into 3 groups. Fourteen rats in Group 1 were injected twice a day for 5 days with PBS and on day 6 were injected intraperineally with an arthropathic dose of sonicated streptococcal cell wall material (PG-PS) containing 20 μg rhamnose/g body weight. They were examined daily and each joint was graded for arthritis on a scale of 0–4.[1] A total score for all 4 feet was recorded and means plus S.D. calculated for the entire group. Six of these 14 rats were killed on the third day after the injection of the PG-PS, at the height of their acute arthritis. Their right feet and livers were assayed for content of PG-PS.[9] Their left feet, liver, and spleen were prepared for histology. The remaining 8 rats were observed for 40 days and killed. The right feet and livers were analysed for PG-PS content.

Thirteen Group 2 rats were injected intraperineally twice a day for 5 days with graded doses of compound 48/80. On day 6 all 13 rats received the same arthropathic dose of PG-PS intraperineally. These rats were observed and their joints scores recorded as before. Seven of the Group 2 rats were killed 3 days after the PG-PS injection and their same tissues studied. The remaining 7 rats in this group were observed for 40 more days and then killed. Their right feet were also analysed for content of PG-PS. The 7 Group 3 rats were used as negative controls. They received the same injections of compound 48/80. Three were killed on day 6 and their feet examined histologically for the presence of granulated mast cells. The other 4 rats were injected with PBS intraperineally on day 6 and killed 3 days later. Their right feet were analysed for PG-PS content.

Results

Morphologic Studies

Sprague-Dawley rats have many mast cells that stain well with toluidine blue after formalin fixation. These cells are particularly prominent in the joints, around tendons, in the skin, ears, nose, and tail. They are present in decreased numbers within the internal organs. Thirty minutes after the intravenous injection of 6 μg (dry wt)/g body weight PS in PBS there was marked swelling of all 4 feet in 11 of 14 rats (Figure 1). Two had slight swelling and 1 had no observable swelling. Four control rats that were injected intravenously with PBS had no swelling. The edema had completely subsided by 6 hours in all rats held for that time.

Toluidine blue stained sections of the feet of rats with edema, killed at 30 minutes, contained many mast cells that had released their granules (Figure 2). The 2 acute test rats that had no or minimal edema

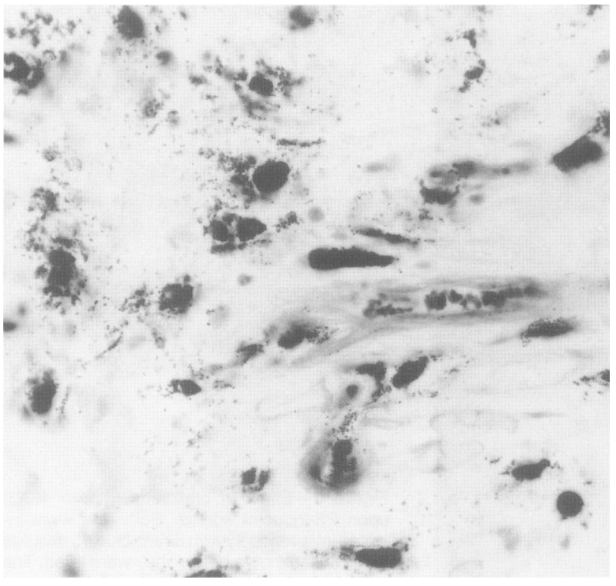

Figure 2—Mast cell release. This toluidine blue stained section of edematous tissues adjacent to a joint in a rat injected 30 minutes earlier intravenously with PS. Most of the dark staining mast cells are surrounded by numerous released granules. (×315)

had only rare mast cells that had released their granules and appeared morphologically indistinguishable from the PBS controls. Significant but less marked granule release was observed in the tissues of the nose and ears. No mast cell changes were observed in the lungs, tail, trachea, spleen, gut, abdominal wall, or thymus. Three of the rats killed 30 minutes after injection, and one acute PBS control also received a dilute suspension of India ink 30 minutes before sacrifice. Two of the 3 test rats that had gross edema demonstrated many venules marked with black carbon particles (Figure 3). These venules were usually adjacent to degranulating mast cells (Figure 4). The labeled vessels were found in the subcutaneous tissues of the feet, adjacent to tendons and within the synovium (Figures 3 and 4). No vessels in other tissues contained carbon particles and none of the venules in the control rats were labeled. The rats killed 6 hours after intravenous PS had no degranulating mast cells and none of their vessels were labeled by carbon particles injected 30 minutes before sacrifice. The 6-hour PBS controls appeared the same.

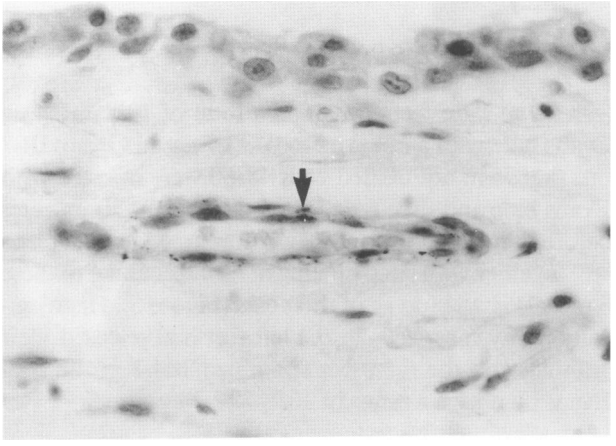

Figure 3—Synovial tissue from the joint of a rat injected intravenously with PS 30 minutes and India Ink 20 minutes before it was killed. The wall of this small vein contains many black carbon particles (arrow). (Kernechtrot's nuclear fast red, ×560)

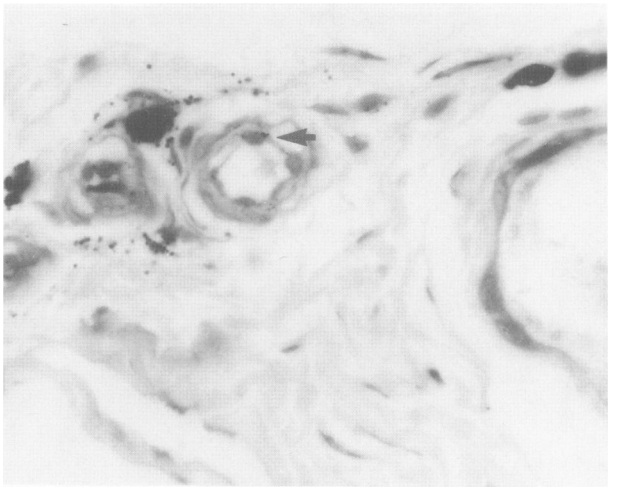

Figure 4—Synovium from the joint of a rat injected intravenously with PS and India Ink 30 minutes before it was killed. The venule near the center is surrounded by dark staining mast cell granules and its wall contains several black carbon particles (arrow). (toluidine blue, ×800)

Effect of Compound 48/80 on PS-Induced Edema

There were 3 experimental groups (Table 1). In Group 1 6 rats were pretreated with 48/80, injected

Table 1—Compound 48/80 Inhibition of Polysaccharide-Induced Edema

Experimental groups	Evans blue dye content in both right feet
I	
Six rats given 48/80 IP for 5 days, day 6 Evans blue 6.5 mg IV 45' and PBS IV 30' before sacrifice	19.1 ± 3.2 μg (range, 17–25)
II	
Seven rats given 48/80 IP for 5 days, day 6 Evans blue 6.5 mg IV 45' and PS IV 30' before sacrifice.	19.5 ± 2.4 μg (range, 16–24)
III	
Seven rats given PBS IP for 5 days, day 6 Evans blue 6.5 mg IV 45' and PS IV 30' before sacrifice.	164 ± 106 μg (range, 36–322)

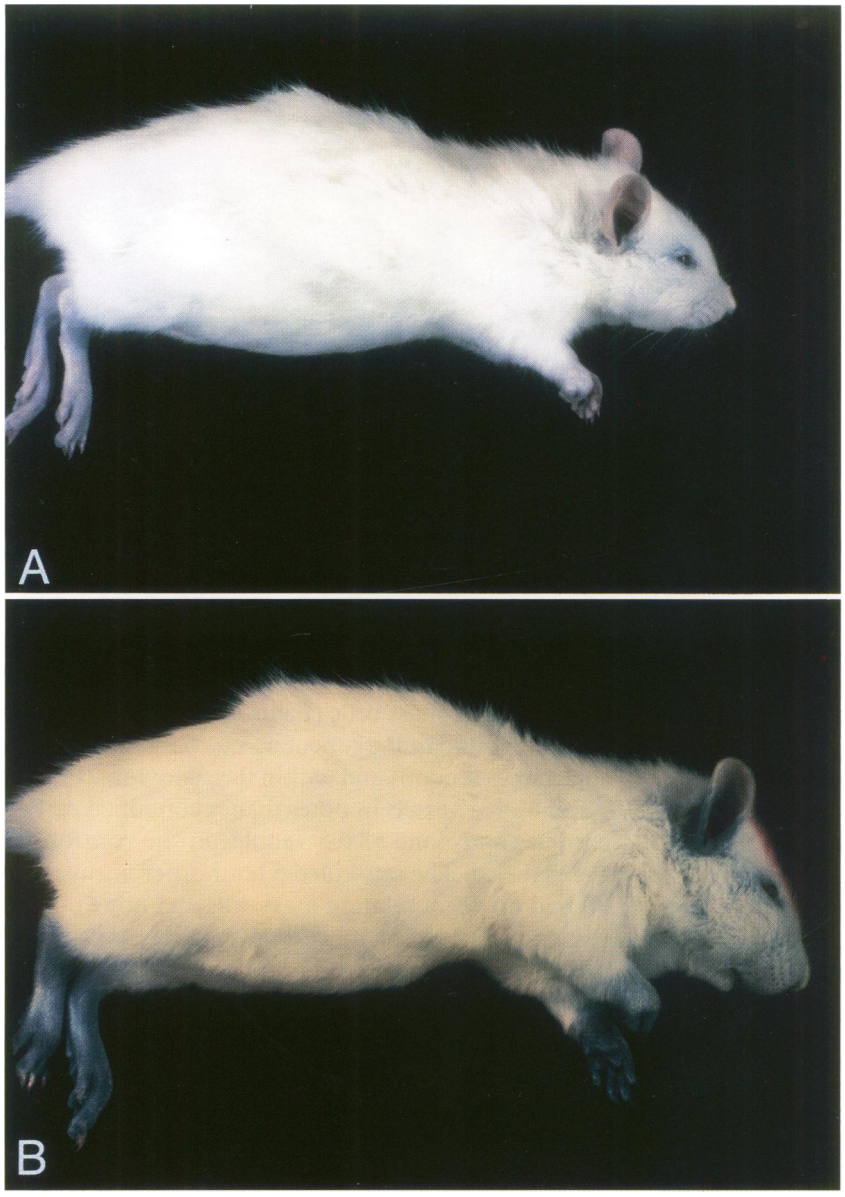

Figure 5—Effect of pretreatment with 48/80 upon PS-induced edema. Both rats were injected intravenously with Evans blue 45 minutes and PS 30 minutes before they were killed. The rat in **A** had been pretreated with 48/80 and had no edema or excess blueing of any tissues. The rat in **B** was not pretreated with 48/80 and quickly developed swollen, blue feet. In addition there was blueing of the nose and the bases of both ears.

with Evans blue and then PBS, and killed after 30 minutes. There was no evidence of edema or increased blueing in any tissues. Histologic sections of the left feet were free of toluidine blue staining mast cells except in the perineurium of peripheral nerves where they remained intact.[8] The right feet contained a total of 19.1 ± 3.2 μg Evans blue with a range of 17.2–25.0 μg. In Group II 7 rats were pretreated with 48/80, injected first with Evans blue and then with 6 μg PS/g body weight 30 minutes before they were killed. None of the rats developed edema and none had increased blueing of any tissues. Histologic sections of the left feet were again free of granulated mast cells except those associated with nerves, and none showed morphologic evidence of granule release. The right feet contained a total of 19.5 ± 2.4 μg Evans blue with a range of 16.0–23.7 μg. In Group III 7 rats that were not treated with 48/80 were injected with Evans blue and then 6 μg PS/g body weight 30 minutes before they were killed. Six rats developed edema (± to 4+) together with deep blueing of all 4 feet and 4 of these 6 rats also had blueing of their ears (Figure 5). Histologically many mast cells in the left feet had released their granules and the right feet contained 164.2 ± 106.0 μg Evans blue with a range of 36.7–322.2 μg.

Effect of 48/80 on PG-PS-Induced Arthritis

The clinical evaluation of the arthritis, shown in Figure 6, indicates that the 48/80 pretreatment had

only slight effect on the arthritis. Half the rats in both groups were killed 3 days after PG-PS injection and the rest were observed and finally killed on day 40. The total PG/PS content of the right feet of the Group 1 rats sacrificed acutely on day 3, 3 days after PG-PS injection, was 2.06 μg (±3.03 μg). The PG-PS content of the right feet of the 48/80 pretreated Group 2 rats killed acutely on day 3 was 1.03 μg (±1.17 μg). The PG-PS content of the right feet of Group 1 rats sacrificed on day 40 was 0.43 μg (±0.41 μg) and that of Group 2 was 0.54 μg (±0.54 μg). None of these differences was statistically significant.

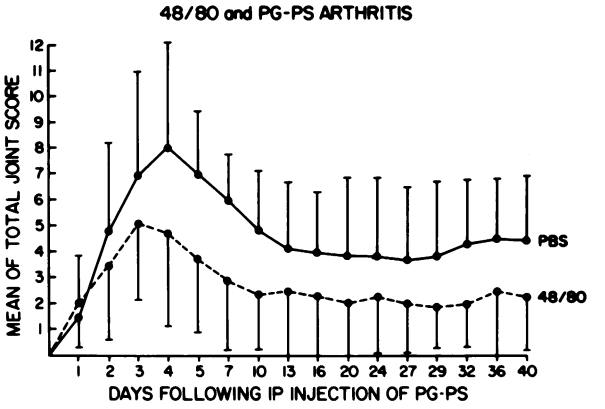

Figure 6—The effect of pretreatment with 48/80 upon PG-PS induced arthritis. The open circles are the total mean joint scores for Group 1 control rats receiving only saline (PBS) before PG-PS and the solid circles represent the total mean scores for the Group 2 rats that were pretreated for 5 days with compound 48/80. The bars are the standard deviations.

Discussion

The data presented here support the conclusion that purified Group A streptococcal PS causes acute edema in the feet of Sprague-Dawley rats by stimulating the local mast cells to release their granules, resulting in increased microvascular permeability and edema. This conclusion is consistent with the acute, transient course of the reaction,[10] the morphologic appearance of the mast cells, the labeling of the adjacent venules with injected carbon particles, and the blocking of the reaction by the pretreatment with compound 48/80. Thus, group A streptococcal PS can probably be added to the growing list of microbial products that cause nonimmune mast cell activation, such as yeast cell wall polysaccharides,[11] staphylococcal cell wall sugars,[12] *E. coli* alpha hemolysin,[13] and staphylococcal enterotoxin B.[14-16]

The presence of large numbers of mast cells in the feet of rats and the selective granule release by purified PS suggested that this early increase in vascular permeability might be the initial reaction responsible for the selective articular deposition of systemically injected PG-PS, because these preparations of cell wall fragments also contain some free PS. However, the degranulation of mast cells with compound 48/80, which blocked the PS-induced acute edema, did not prevent either the deposition of PG-PS in the joints or the development of arthritis. Moreover, toluidine blue stains of the joints of rats injected intraperineally with an arthropathic dose of PG-PS from an earlier study[2] failed to reveal any evidence of mast cell release during the early hours after injection. Another explanation for the selective deposition of PG-PS in experimental arthritis probably lies in a related observation from this laboratory. When PG-PS polymer from group A streptococci is solubilized by M-1 mutanolysin it is no longer arthropathic, but these small molecular weight fragments of PG-PS will induce paw edema when injected either intravenously[4] or directly into joints.[17] However, this reaction persists for as long as 12 hours.[4] The increased permeability caused by soluble PG-PS is thus an immediate-prolonged reaction,[10] suggesting that it is due to endothelial injury, direct or indirect, not mediated by local histamine release. A preliminary study of edema induced by small molecular weight (mutanolysin degraded) PG-PS, demonstrated carbon labeling of articular venules without morphologic evidence of mast cell release. Because the sonicated PG-PS preparations also contain some small molecular weight PG-PS, it is hypothesized that the systemic injection of these small fragments in these preparations causes the initial vascular injury that then leads to the articular localization of the larger arthropathic fragments. Thus, both free PS and small PG-PS polymers can increase vascular permeability and contribute to the localization of arthropathic PG-PS in the limbs, but only the PS functions through mast cell degranulation. Just why the vascular bed of the distal joints in the rat is more sensitive to this injury is not yet understood, but there are other examples of the selective effects of systemically injected bacterial products on specific vascular beds.[18-20]

In another context, the degranulation of mast cells by streptococcal PS might explain why bacteremia in humans can result in infectious arthritis.[21] Many other bacteria probably produce constituents capable of activating mast cells and because human joints contain large numbers of these cells,[22] degranulation and transient vascular leakage could be involved in the pathogenesis of articular infections.

References

1. Cromartie WJ, Craddock JG, Schwab JH, Anderle SK, Yang CH: Arthritis in rats after systemic injection of

streptococcal cells or cell walls. J Exp Med 1977, 146: 1585–1602
2. Dalldorf FG, Cromartie WJ, Anderle SK, Clark RL, Schwab JH: The relation of experimental arthritis to the distribution of streptococcal cell wall fragments. Am J Pathol 1980, 100:383–402
3. Chetty C, Brown RR, Schwab JH: Edema-producing activity of group A streptococcal polysaccharide and its possible role in the pathogenesis of cell wall-induced polyarthritis. J Exp Med 1983, 157:1089–1100
4. Chetty C, Klapper DG, Schwab JH: Soluble peptidoglycan-polysacchariedfragments of bacterial cell wall induced acute inflammation. Infect Immun 1982, 38: 1010–1019
5. Krause RM, McCarty M: Studies on the chemical structure of the streptococcal cell wall. I. The identification of a mucopeptide in the cell walls of groups A and A-variant streptococci. J Exp Med 1961, 114:127–140
6. Dische Z, Shettles LB: A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J Biol Chem 1948, 175:595–603
7. Udaka K, Takeuchi Y, Movat HZ: Simple method for quantitation of enhanced vascular permeability. Proc Soc Biol Med 1970, 133:1384–1387
8. Riley JF, West GB: Tissue mast cells: Studies with histamine-liberator of low toxicity (compound 48/80). J Pathol Bact 1955, 69:269–282
9. Eisenberg R, Fox A, Greenblatt JJ, Anderle SK, Cromartie WJ, Schwab JH: Measurement of bacterial cell wall in tissues by solid phase radioimmunoassay: Correlation of distribution and persistence with experimental arthritis in rats. Infect Immun 1982, 38:127–135
10. Ryan GB, Majno G: Acute inflammation, a review. Am J Pathol 1977, 86:185–276
11. Poyser RH, West GB: Changes in vascular permeability produced in rats by dextran, ovomucoid and yeast cell wall polysaccharides. Br J Pharmacol 1965, 25:602–609
12. Jensen C, Stahl Skov P, Norn S, Espersen F, Bog-Hansen TC, Lihme A: Complexity of lectin-mediated reactions in bacteria-induced histamine release. Allergy 1985, 39:451–456
13. Konig B, Konig W, Scheffer J, Hacker J, Goebel W: Role of Escherichia coli alpha-hemolysin and bacterial adherence in infection: Requirement for release of inflammatory mediators from granulocytes and mast cells. Infect Immun 1986, 54:886–892
14. Scheuber PH, Golecki JR, Kickhofen B, Scheel D, Beck G, Hammer DK: Skin reactivity of unsensitized monkeys upon challenge with staphylococcal enterotoxin B: A new approach for investigating the site of toxin action. Infect Immun 1985, 50:869–876
15. Scheuber PH, Denzlinger C, Wilker D, Beck G, Keppler D, Hammer DK: Staphylococcal enterotoxin B as a nonimmunological mast cell stimulus in primates: The role of endogenous cysteinyl leukotrienes. Int Arch Allergy Appl Immun 1987, 82:289–291
16. Bamberger U, Scheuber PH, Sailer-Kramer B, Bartsch K, Hartmann A, Beck G, Hammer DK: Anti-idiotypic antibodies that inhibit immediate-type skin reactions in unsensitized monkeys on challenge with staphylococcal enterotoxin. Proc Natl Acad Sci USA 1986, 83:7054–7058
17. Esser RE, Anderle SK, Chetty C, Stimpson SA, Cromartie WJ, Schwab JH: Comparison of inflammatory reactions induced by intraarticular injection of bacterial cell wall polymers. Am J Pathol 1986, 122:323–334
18. Dalldorf FG, Beall FA, Krigman MR, Goyer RA, Livingston HL: Transcellular permeability and thrombosis of capillaries in anthrax toxemia: An electron microscopic and biochemical study. Lab Invest 1969, 21:42–51
19. Shaffer MF, Bennett GA: The passage of type III rabbit virulent pneumococci from the vascular system into joints and certain other body cavities. J Exp Med 1939, 70:293–302
20. Lewis GW, Cluff LE: Synovitis in rabbits during bacteremia and vaccination. Bull Johns Hopkins Hosp 1965, 116:175–190
21. Goldenberg DL, Reed JI: Bacterial arthritis. N Engl J Med 1985, 312:764–771
22. Janes J, McDonald JR: Mast cells: Their distribution in various human tissues. Arch Pathol 1948, 45:622–634