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Sequential Events in the Pathogenesis of Streptococcal Cell Wall-induced Arthritis and Their Modulation by Bis(5-amidino-2-benzimidazolyl)methane (BABIM)

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This report builds on the authors' earlier discovery of bis(5-amidino-2-benzimidazolyl)methane (BABIM) as a strong suppressive agent for streptococcal cell wall fragment-induced arthritis in the Lewis rat. As a synthetic inhibitor of trypsinlike proteases, BABIM opens up a new route to the control of inflammatory joint disease. To gain a deeper insight into the function of the compound, the authors have now studied its influence on the sequential development of the joint changes and the associated lesions in spleen and liver. Bis(5-amidino-2-benzimidazolyl)methane is shown to block acute synovitis, to retard and reduce granuloma formation in spleen and liver, to decrease neutrophilic leukocytosis, and to diminish hemopoietic hyperplasia in the bone, and thus also to mitigate the distinctive osteoclastic and chondroclastic events. The compound does not interfere with the splenic immune response, the temporary rise in hepatocytic mitotic activity, or the organ deposition of streptococcal cell walls. (Am J Pathol 1990, 136: 909–921)

Previously, we have shown that treatment with the potent protease inhibitor bis(5-amidino-2-benzimidazolyl)methane (BABIM) can improve significantly the clinical signs and morphologic abnormalities of experimental arthritis in the Lewis rat.[1] In that study, inflammation was induced by systemic application of streptococcus group A cell-wall–derived peptidoglycan polysaccharide (PG-APS) and all animals were killed at the same time, 19 days after initiation of the arthritis. Animals treated with intravenous application of BABIM had a striking reduction in the severity of their synovitis and their destructive lesions in ankle and foot bones. There also was a marked decrease in the number of granulomata, which are found in the liver of Lewis rats as a characteristic response to PG-APS. Furthermore, it was noted that osteitis was always accompanied by replacement of the fatty marrow by trilineage hemopoietic hyperplasia, although it remained unresolved if inflammation preceded hyperplasia or vice versa. Regardless of the actual sequence, BABIM mitigated both those processes.

Bis(5-amidino-2-benzimidazolyl) is a strong inhibitor of trypsin, thrombin, plasmin, and urokinase.[2,3] Inhibition of those enzymes or of related arginyl- and lysyl-directed esteroproteases is held responsible for its ability to block replication of respiratory syncytial virus and rotavirus and to reduce tumor invasion and metastases.[4–8] As the drug now also offers a new approach to the treatment of arthritis and holds considerable promise for use in humans, it becomes imperative to investigate in greater detail the histologic aspects of the arthritis model. To this end, the progress of the disease and its modulation by BABIM have now been followed at close time intervals, and special attention has been directed toward the development of lesions in synovium, bone, spleen, and liver and toward changes in the circulating white blood cell count. The results, as presented here, have made it possible to arrange the pathologic events in a predictable sequence and to pinpoint the sites of interference of BABIM with those processes.

Materials and Methods

Streptococcal Cell Wall Fragments

The material was prepared as previously described in detail.[9] In brief, group A beta-hemolytic streptococci (type 3,

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strain D-58) were harvested and washed by continuous-flow centrifugation. The cells were disrupted in a Braun homogenizer. The cell walls were separated by differential centrifugation, purified by repeated extraction with 2% sodium dodecyl sulfate and then dialyzed and freeze-dried. The purified cell walls were suspended in phosphate-buffered saline, dispersed by sonication, and centrifuged at 10,000*g* for 30 minutes. The fragments in the supernatant were filtered through a 0.45-μ Millipore membrane and injected intraperitoneally.

Quantitation of Streptococcal Cell Wall in Tissue

An established enzyme-linked immunosorbent assay was used to quantitate the amount of PG-APS in rat liver and spleen.[10] Tissue samples were homogenized in TRIS buffer for 30 seconds with a Sonifier Cell Disruptor (Branson Sonic Power Group). The samples were microfuged for 2 minutes in a Fisher microcentrifuge, and supernatants of the extracts were assayed. The extracts, after suitable dilution, were added to 96-well microtiter plates previously coated with affinity-purified anti-group A antibody. Biotinylated affinity-purified anti-group A antibody then was added, followed by avidin-alkaline phosphatase. The color reaction was developed using p-nitrophenyl phosphate substrate, and the optical density was determined on an automated 96-well microplate reader (Molecular Devices).

Bis(5-amidino-2-benzimidazolyl)methane (BABIM)

Synthesis of the inhibitor has been described in an earlier publication.[2] Purity of the compound was greater than 99.6%. The formula of the preparation used was $C_{17}H_{16}N_4/4.3$ HCl/1.7 H_2O, including 64% by weight of the active principle. For injection into the rats, solutions of the material in isotonic saline were prepared fresh on a daily basis.

Treatment of Animals

Inbred female Lewis rats weighing 145 g were obtained from Charles River Breeding Laboratories (Wilmington, MA). They had unlimited access to regular laboratory chow and water. Arthritis was initiated by a single intraperitoneal injection of 0.5 ml of the streptococcal cell wall material (PG-APS) described above. The amount of PG-APS given to each animal was equal to 15 μg of rhamnose content per gram of body weight. Half the number of animals received daily injections of BABIM dissolved in 0.5 ml saline, while the other half received saline only. Application was into the tail vein under light ether anesthesia.

Histologic Methods

Death of the animals was by an overdose of ether. Immediately afterwards, the liver and spleen were removed and weighed. Aliquots of each organ were frozen and saved for PG-APS determination, while the rest was fixed in buffered formalin. The hind legs were skinned and were also submitted. Following fixation, they were decalcified in formic acid-sodium citrate solution. All tissues were embedded in paraffin and stained with hematoxylin and eosin as well as with periodic acid-Schiff (PAS) after diastase digestion. To determine the density of the granulomata in the liver, it was necessary to measure the area of the liver sections. This was done with the help of a Zeiss Videoplan computer image analyzer (Carl Zeiss, West Germany).

Clinical Evaluation of Arthritis

The extent of the arthritic process was judged on a daily basis and scored as a number.[11] Involvement of front and hind legs was determined and rated for each extremity on a scale of 0 to 4, making possible a maximal grade of 16. Only observations on the wrist and ankle joints and the tissues distal from there were taken into consideration. Evaluation involved the degree of cutaneous erythema and soft tissue edema as well as the severity of joint swelling and deformity.

Histologic Scoring of Arthritis

To rank the histologic changes, attention was centered on the synovium of the talocrural joint and on the distal tibia and the calcaneus. In the synovium, the following features were looked for; lining cell hyperplasia, edema, fibrin deposits, acute and chronic inflammatory cells, joint exudate, and capillary and fibroblastic proliferation. In the bones, evaluation was focussed on the degree of hemopoietic hyperplasia, osteoclasia and chrondroclasia, neutrophilic infiltration, fibrosis and new bone formation, and the destruction of cortical bone, epiphyseal plates, and joint cartilage. The highest attainable score was 15.

White Blood Cell Count

Total white blood cell counts were determined with the help of a Baker cell counter (Baker Instruments) on heart

blood anticoagulated with EDTA. White blood cell differential counts were carried out on blood smears stained with Wright's stain. A total of 100 cells were counted and the types were identified by morphology.

Statistical Analysis

Student's *t*-test was employed to evaluate the statistical significance of the difference between two means.

Results

The experimental approach can be briefly summarized as follows: Lewis rats with PG-APS–induced disease received a daily intravenous injection of either BABIM or of the saline vehicle. The dose was 10 mg/kg body weight of the preparation specified above. Administration of the compound began 2 days before the induction of arthritis and ended 24 hours before death. For logistical reasons, the experiment was run in two stages, the first furnishing data from day 0 through day 5 and the second from day 6 through day 14. As all conditions were closely matched, there was a nearly seamless transition between the two sets of data.

Joints and Bones

The clinical and the histologic severity of the joint disease is demonstrated in Figure 1A and Figure 1B, respectively. The clinical scores represent the mean values for all treated or untreated animals remaining on a given day, while the histologic scores represent the mean values of those treated or untreated animals killed on a given day. The animals were usually killed in groups of four. The only exception was at day 14, when there were five animals to either group. From the graphs, it is evident that inflammation in the control animals was well established by day 1 and that it became more pronounced as time progressed. The figures also make it clear that BABIM was remarkably effective in mitigating the pathologic events throughout the length of the experiment.

Histologically, the first changes in the BABIM-free animals appeared in the synovium of the talocrural joint and then spread to the other, more distal joints of the foot. In the day 1 specimen from the ankle joint, there were noted synovial lining cell hypertrophy and hyperplasia, fibrin deposition on top of the lining cells and on areas of lining cell loss, and a scant neutrophilic infiltrate in the stroma. The synovial recess was dilated and the lumen contained small numbers of neutrophils. At day 2, neutrophils had become more prominent in stroma and joint recess. There

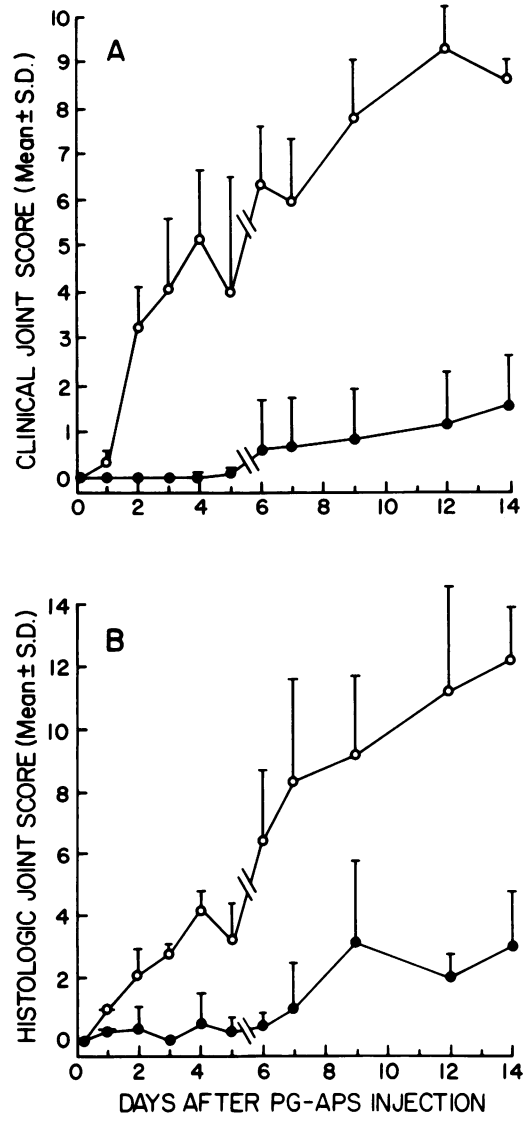

Figure 1. *Clinical (A) and histologic (B) joint scores in BABIM-treated (-●-) and vehicle-treated (-○-) Lewis rats followed for 2 weeks after PG-APS injection. At 24 hours and at all points beyond, the differences between the two series were statistically significant ($P < 0.05$) in both scoring systems.*

was dilatation of venules and capillaries and marked soft tissue edema. Small groups of lymphocytes were observed for the first time. Many of the hyperplastic synovial cells now showed cytoplasmic vacuolization. At days 3 and 4, there was the added feature of fibroblastic and capillary proliferation. Edema had decreased at day 5, as had the number of neutrophils. Fibroblasts began to lay down collagen. At day 6, stromal cell proliferation persisted and small numbers of lymphocytes were still encountered. By day 9, neutrophils began to make a comeback, and they achieved their most massive infiltration on days 12 and 14. It should be added that similar findings as those just described for the joint synovium were en-

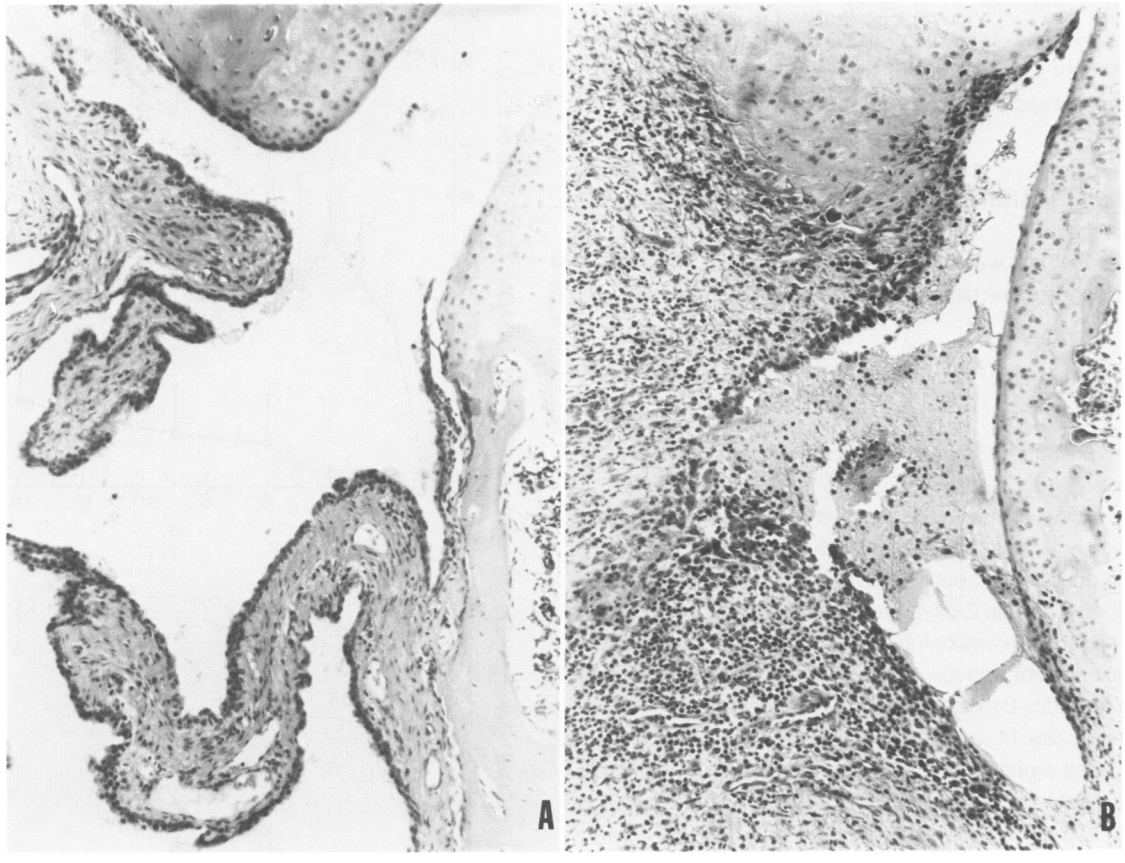

Figure 2. *Synovium of talocrural joint in Lewis rats 14 days after systemic PG-APS injection.* A: *Absence of inflammation in BABIM-treated animal.* B: *Marked acute inflammation in vehicle-treated animal (H&E, ×100).*

countered also in the tendon sheaths of the lower leg and the bursa of the Achilles tendon.

A comparison of the synovial changes in BABIM-treated animals with those in the controls confirmed the suppressive activity of the compound (Figure 2). From all aspects of PG-APS–induced disease, synovitis seems to be the condition most susceptible to inhibition. In the majority of treated rats, inflammation was completely absent, while in the others its severity was greatly reduced. In the latter cases, it appears that none of the various histologic expressions of the inflammatory process in the synovium, such as lining cell hyperplasia, edema, or neutrophilic infiltration, was more effectively countered than any other.

After synovitis, the next most important pathologic abnormality in the hind legs was related to the bones themselves. In the untreated animals, there was a stereotyped unique set of events that began in the distal tibia and spread within a week to also involve the tarsal and metatarsal bones. The initial occurrence, starting 3 days into the experiment, was an increase in hemopoietic elements in the marrow cavity of the distal tibial diaphysis, and in the metaphysis and epiphysis. Fat cells, which normally make up 80% or more of the marrow, were being replaced by erythroid, myeloid, and megakaryocytic cell lines in all stages of maturation (Figure 3). At day 9, complete replacement had been achieved. As early as day 3, and always in association with increased hemopoietic activity, there was the appearance of osteoclasts within the metaphyseal region. The cells were arranged singly or in clusters and were commonly accompanied by groups of mature neutrophils. The neutrophils often were closely packed into Howship's lacunae together with the osteoclasts (Figure 4). After 7 days, osteoclasia was seen in all animals, and it proceeded at a rapid pace to destroy cortical as well as medullary bone. Epiphyseal bone also became victim of the onslaught, and finally there was dissolution of the epiphyseal plate from both its sides and erosion of the joint cartilage from the subchondral marrow spaces (Figures 5B and C). In the areas of active osteoclasia, hemopoietic elements usually disappeared and left behind the fibrovascular framework with an infiltrate of varying numbers of mature neutrophils. Finally, after completion of the bone destruction, reparative processes came into play, consisting of stromal proliferation and new bone formation. Proliferation also was encountered in the periosteum, often associated with osteoblastic activity.

Under the influence of BABIM treatment, there was a delay of the appearance of hemopoietic hyperplasia of

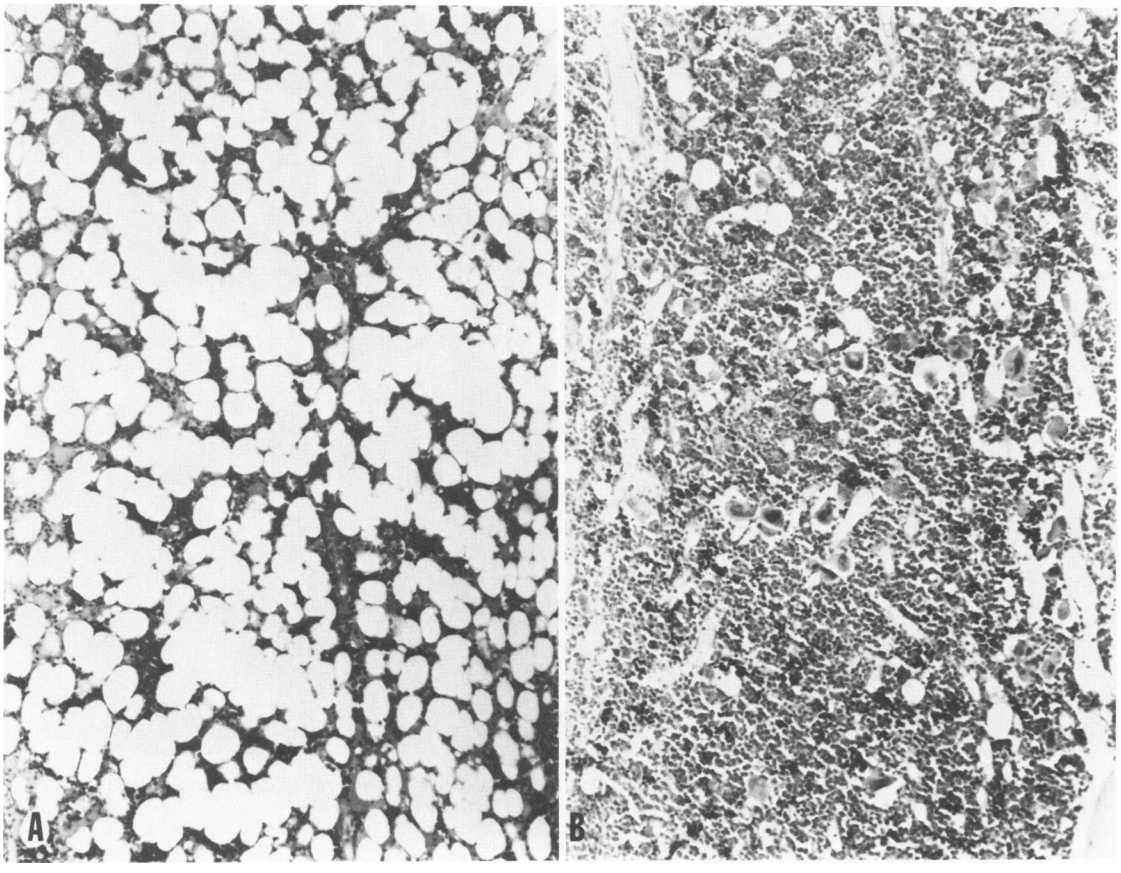

Figure 3. *Bone marrow cavity in distal tibial shaft in Lewis rats 7 days after PG-APS injection.* **A:** *Predominantly fatty marrow in BABIM-treated animal.* **B:** *Marked trilineage hemopoietic hyperplasia in vehicle-treated animal (H&E, ×100).*

about 3 days, and there also was a delay and significant amelioration of all the other pathologic changes described for the bones (Figure 5A). Numerically, the degree of improvement by BABIM is best reflected by the histologic scores in Figure 1B, where 11 points out of a possible 15 were allocated to rank the various bone lesions.

Spleen

The first histologic changes in the spleen, as observed at 6 hours after PG-APS injection, consisted of deposits of PAS-positive material, presumably streptococcal cell wall fragments.[12] The material appeared finely granular and was located in macrophages in the marginal zone and in the red pulp. Subsequent to its accumulation, three distinct reaction patterns evolved: a granulomatous process, a non-specific immune response, and a proliferation of hemopoietic elements. Granulomata were in evidence as early as at 24 hours. They could be recognized as aggregates of typical epithelioid cells in the marginal zone, where they became associated with increasing numbers of neutrophils. Number and size of the granulomata rapidly increased and large central areas of necrosis developed. At 9 days, 60 to 80% of the white and red pulp were destroyed and the initially spherical shape of many of the granulomata had changed into an irregular geographic pattern (Figure 6). The ramifying central stretches of nonviable tissue were replete with neutrophils. They were surrounded by epithelioid cells and histiocytes, and those again were enveloped by fibroblasts. Beyond day 9, collagen was being laid down at the periphery of the granulomata, and the centers appeared to shrink because of to removal of the necrotic debris. This led to a relative increase in nongranulomatous parenchyma, so that the histologic sections looked less abnormal than they had just a few days earlier. Ascendancy and decline in granulomatous involvement are reflected in the remarkable rise and fall of the spleen weights, as shown in Figure 7. As is to be expected with any injection of antigenic foreign material, the spleen also developed an acute immune response. At day 4, immunoblasts and plasma cells were found clustered in the red pulp, and at days 6 and 7 they were very prominent. There also was a brief appearance of germinal centers, many of them distinguished by tangible body macrophages.

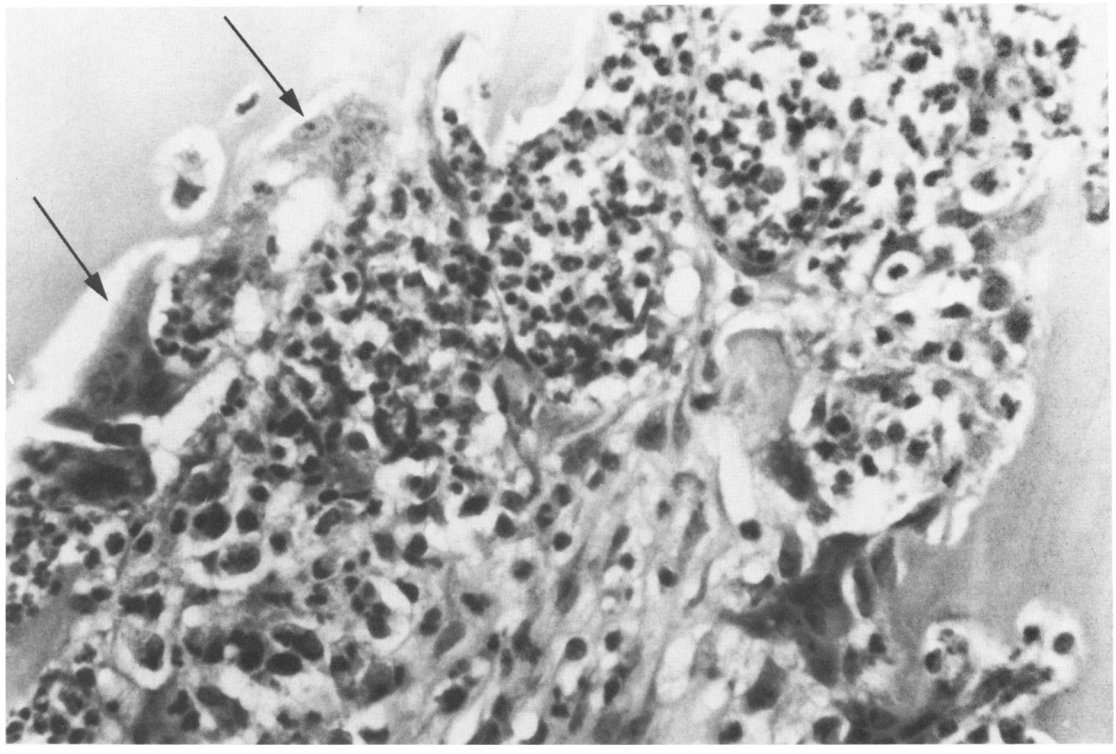

Figure 4. *Neutrophil-assisted osteoclasia in distal tibial metaphysis in vehicle-treated Lewis rat 14 days after PG-APS injection. Characteristic grouping of neutrophils with osteoclasts (arrows) in Howship's lacunae (H&E, ×540).*

The plasma cell infiltration came to a rather precipitous end, when at day 9 all the red pulp became solidly packed with hemopoietic elements of the erythroid, myeloid, and megakaryocytic cell lines. This situation remained unchanged through the final day of the experiment.

An evaluation of the various splenic responses in vehicle-treated and BABIM-treated animals brought to light one significant difference. This was a delay in the appearance of the granulomata in the latter series and a reduction in their number, as determined from counts on whole transverse sections of the spleens (Table 1). Beyond day 5, however, granulomatous involvement evened out between the two series. Immune reactivity, as expressed by plasma cell and lymphocytic infiltrates in the red pulp, and degree of hemopoiesis were not affected by the drug. There was no influence of BABIM on the spleen weights (Figure 7).

Liver

In the liver, there occurred two PG-APS–related events that deserve attention; a burst in mitotic activity and the formation of granulomata. An increased rate of division in the hepatic parenchymal cells was detected during routine examination of the histologic sections, and the initial impression was confirmed by quantitation. For each liver specimen, the total number of mitoses in 40 consecutive high-power fields (× 450) was established. As is demonstrated in Figure 8, the mitotic count became sharply elevated at days 6, 7, and 9 and then returned to normal. It is also evident that the liver weights rose in concert with the mitotic activity, suggesting that parenchymal hyperplasia probably was responsible for the weight gain. It should be noted that the mitotic figures seen were from all phases of the replication cycle. This makes it unlikely that mitotic "arrest" was producing a false impression of increased mitotic activity. Finally, the closeness of the mitosis data for BABIM-treated and control animals allows the conclusion that cell division was not affected by the test compound.

Periodic acid-Schiff–positive material appeared in the periportal Kupffer cells of the liver as early as it did in the macrophages of the spleen, ie, at 6 hours after PG-APS injection. However, the granulomatous reaction took a week longer to express itself in the liver than in the spleen. Only at day 12 was the process well under way. Granulomata developed preferentially in the portal areas, but some also originated around central or sublobular veins or within the lobules. The lesions began as small groups of epithelioid cells surrounded by a rim of lymphocytes, scattered neutrophils, and PAS-positive non-Kupffer cell

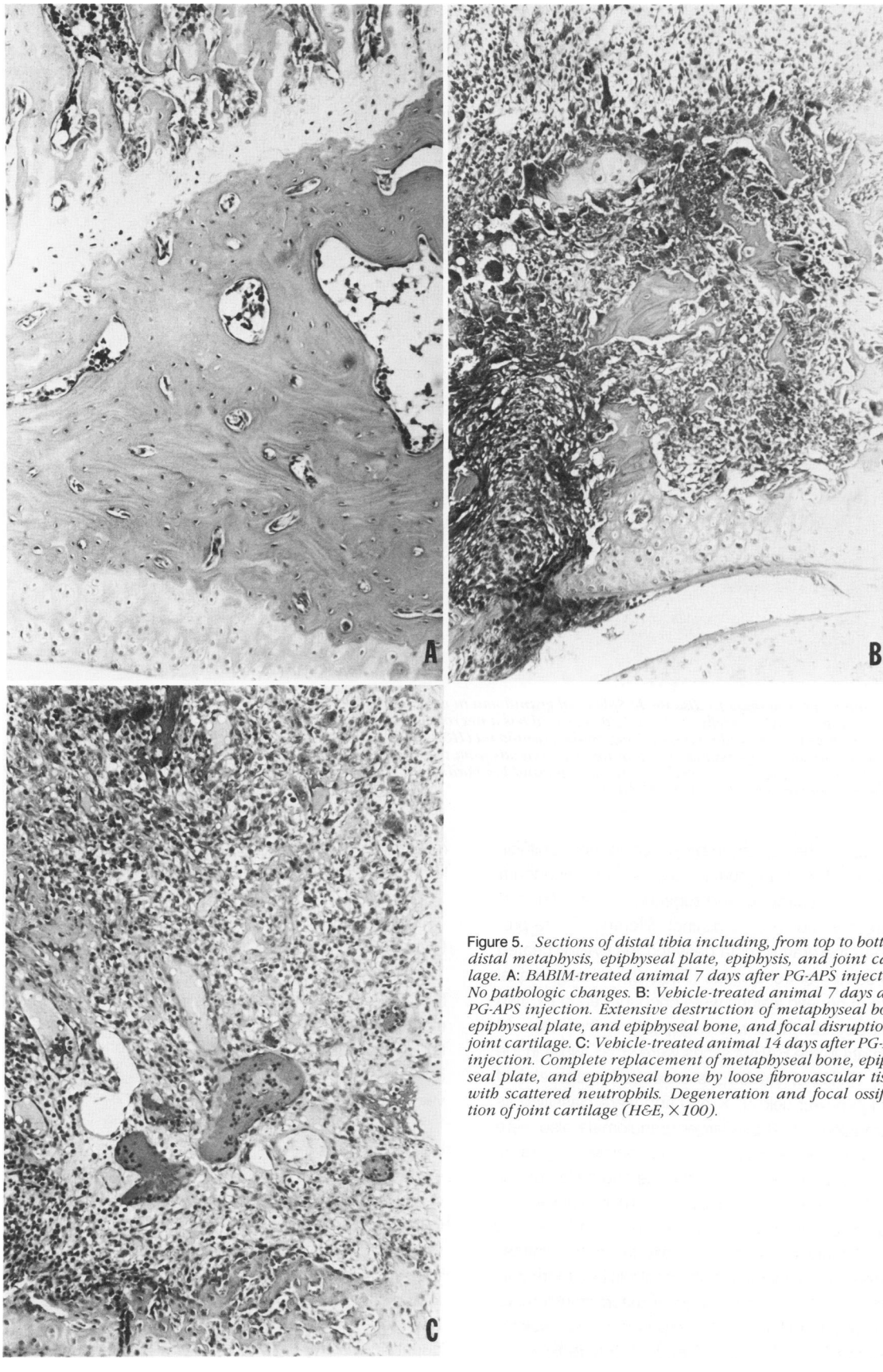

Figure 5. *Sections of distal tibia including, from top to bottom, distal metaphysis, epiphyseal plate, epiphysis, and joint cartilage.* **A:** *BABIM-treated animal 7 days after PG-APS injection. No pathologic changes.* **B:** *Vehicle-treated animal 7 days after PG-APS injection. Extensive destruction of metaphyseal bone, epiphyseal plate, and epiphyseal bone, and focal disruption of joint cartilage.* **C:** *Vehicle-treated animal 14 days after PG-APS injection. Complete replacement of metaphyseal bone, epiphyseal plate, and epiphyseal bone by loose fibrovascular tissue with scattered neutrophils. Degeneration and focal ossification of joint cartilage (H&E, ×100).*

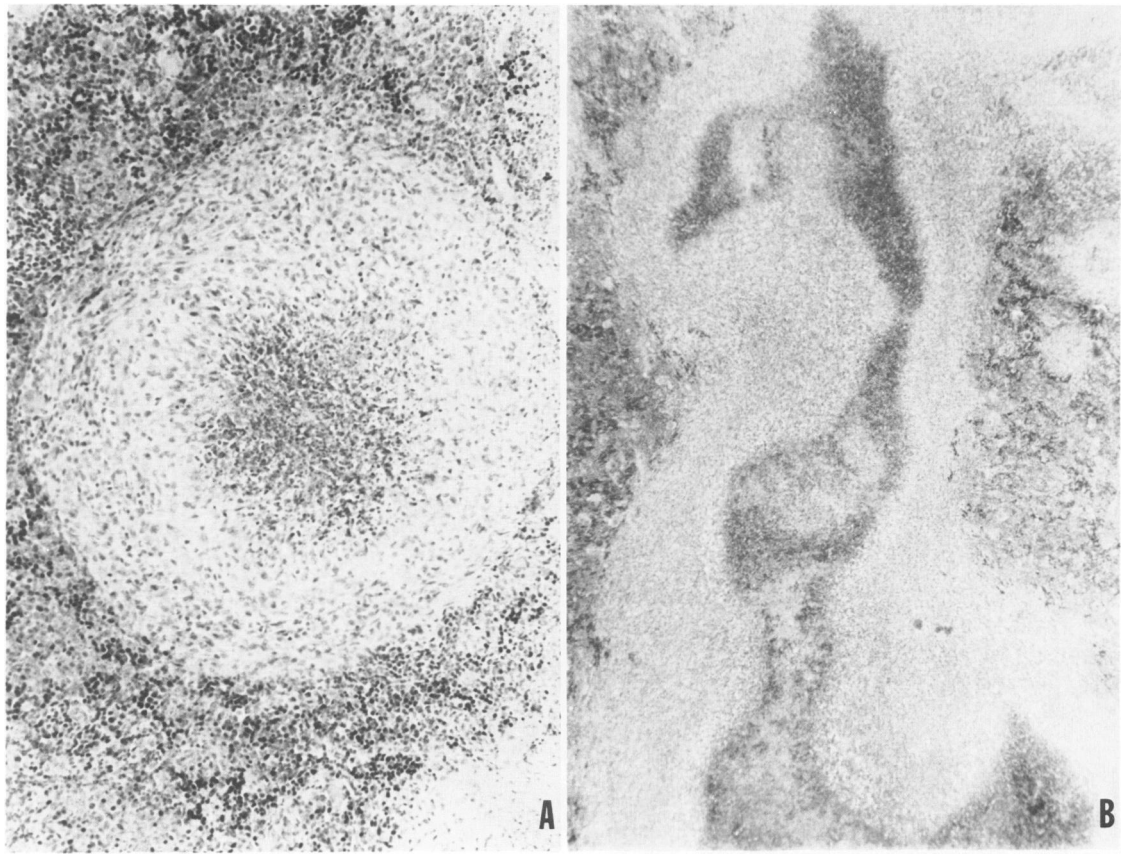

Figure 6. *Splenic granulomatous disease.* **A:** *Spherical granuloma in vehicle-treated rat 14 days after PG-APS injection. The sharply demarcated lesion consists chiefly of epithelioid cells and has a necrotic center containing degenerating neutrophils. Hemopoietic elements are present in the red pulp bordering on the granuloma (H&E, ×100).* **B:** *Confluent necrosis in vehicle-treated rat 12 days after PG-APS injection. An irregular band of necrosis extends from top to bottom through the center of the field and is framed by layers of pale-staining epithelioid cells. Along the right and left borders of the field there are small amounts of preserved red pulp infiltrated by hemopoietic cell nests (H&E, ×40).*

macrophages. They enlarged by acquiring more epithelioid cells, and beyond a certain size they experienced a heavy influx of neutrophils and subsequent central necrosis. There was, however, a distinct difference in the progression of the granulomata between the BABIM-treated and the saline control animals (Table 2). Although equal numbers of hepatic lesions were encountered in the two series at day 12, there was a disparity in their size. All granulomata in the BABIM series were small, while in the controls, 17.4% of the lesions exceeded a diameter of [fr1/2] high-power field at × 450 and were classified as macrogranulomata. Those larger granulomata also were the ones with central neutrophils and necrosis (Figure 9). At day 14, the difference between the two series of animals had become more pronounced. There still were no macrogranulomata in drug-exposed rats, and the total number of granulomata had actually fallen. In contrast, the number of granulomata in the controls had more than doubled, as had the percentage of macrogranulomata. It is likely that the increasing divergence of liver weights between the series at days 12 and 14 (Figure 8) was a reflection of the different degrees of severity of the granulomatous involvement.

Circulating White Blood Cells

Injection of PG-APS into the Lewis rats provoked a marked leukocytosis. Neutrophils and monocytes experienced a greater relative increase than lymphocytes, but in absolute numbers all three cell lines reached similar peak values (Figure 10).

In the lymphocyte series, the counts rose continuously during the first week and leveled off during the second week. Shape and height of the graphs were not significantly different between the vehicle-treated and the BABIM-treated groups (Figure 10A). Monocyte numbers peaked between 7 and 9 days and tended to fall during the second week (Figure 10B). While the fall was unbroken for the drug-treated series, the untreated series rebounded between 12 and 14 days and ended up with a count significantly higher than the BABIM-treated animals.

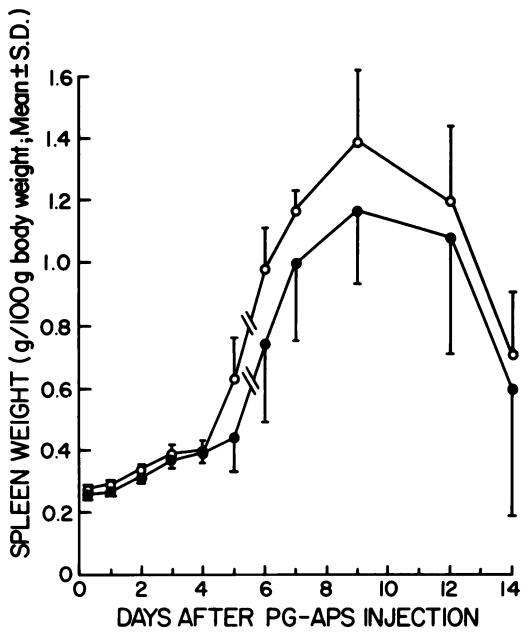

Figure 7. *Spleen weights in BABIM-treated (-●-) and vehicle-treated (-○-) rats followed for two weeks after PG-APS injection. All groups consisted of four animals, except for the day-14 groups which were made up of five animals. The differences between the two series of animals were not statistically significant (P > 0.05).*

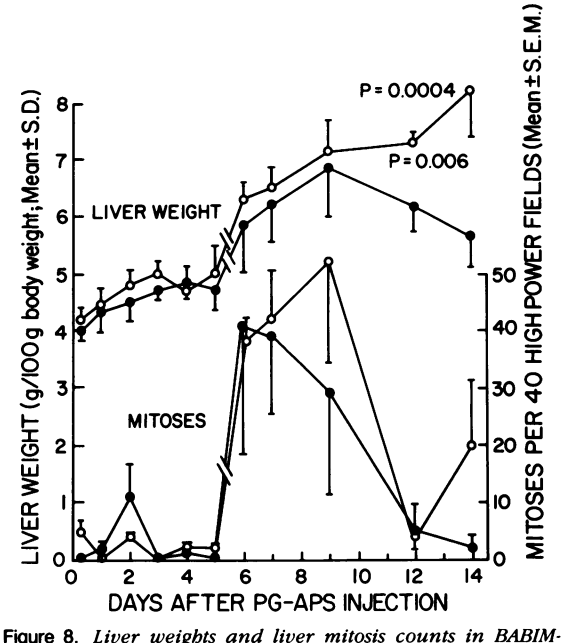

Figure 8. *Liver weights and liver mitosis counts in BABIM-treated (-●-) and vehicle-treated (-○-) rats followed for 2 weeks after PG-APS injection. All groups consisted of four animals, except for the day-14 groups which were made up of five animals. Liver weights were significantly different between the two series on days 12 and 14. For the mitosis count, the day-7 value for the BABIM-treated animals and the day-6, day-7, and day-9 values for the vehicle-treated animals were significantly different (P < 0.05) from the preceding day-3, day-4, and day-5 values.*

With regard to the neutrophils, the untreated animals showed a progressive increase through both weeks, while the treated animals demonstrated a continuous decline after the first week (Figure 10C). At 14 days, the difference between the two sets of animals was highly significant.

Deposition of PG-APS in Spleen and Liver

To determine if differences in the deposition of streptococcal cell wall fragments could have accounted for the different histologic lesions observed in BABIM-treated and vehicle-treated animals, amounts of PG-APS were measured in liver and spleen. On examining the results in Figure 11, it is evident that there was no significant discordance between the findings in the two series of animals.

As expected from the histologic studies, organ deposition of PG-APS was well under way at 6 hours after injection of the material. In the spleen, the values peaked after 2 days and leveled off during the second week. In the liver, the values for the BABIM-treated animals also reached their highest level at 2 days, while the vehicle-treated animals had already experienced their peak a day earlier. As the size of the liver deposits for the two series of rats was well matched at all other points, the importance of the initial discrepancy remains uncertain at this point. Future studies may reinvestigate this aspect for confirmation. However, it is interesting that the PG-APS deposits in the liver rose to a second peak early in the second week of the experimental period. One wonders if the early steps in the digestion of PG-APS by Kupffer cells could have

Table 1. *Effect of BABIM on the Occurrence of Splenic Granulomata in the Early Post-PG-APS Injection Period*

	Number of granulomata per splenic section†				
	Days after injection of PG-APS				
Treatment*	1	2	3	4	5
BABIM	0	0	0.13 ± 0.25	0.25 ± 0.5	2.0 ± 3.02‡
Saline	0	1.38 ± 1.2	0.75 ± 0.87	2.60 ± 2.40	7.5 ± 3.43

* n = 4 for either group at each point.
† Granulomata were counted in two spleen sections per animal. Values are means ± SD.
‡ $P < 0.05$ against saline control.

Table 2. *Effect of BABIM on the Total Number of Granulomata and the Percentage of Macrogranulomata in Sections of Liver*

	Days after PG-APS injection			
	12		14	
Treatment*	Granulomata (no./100 mm²)	Macrogranulomata (% of total)	Granulomata (no./100 mm²)	Macrogranulomata (% of total)
BABIM	40.5 ± 23.9	0	30.4 ± 8.5†	0
Saline	47.4 ± 21.3	17.1 ± 9.8	107.5 ± 66	34.3 ± 9.3

* For the day-12 group, n = 4; for the day-14 group, n = 5.
† Significantly different from the saline group ($P < 0.005$). Values are means ± SD.

unmasked additional reactive epitopes and thus produced a rebound in the ELISA assay.

Comparing the total amounts of PG-APS in the two organs in Figure 11 makes it obvious that there were larger deposits in the liver than in the spleen. As the livers were more than 10 times heavier than the spleens, however, the concentration of PG-APS in the spleens was considerably higher than in the livers. This may have been an additional factor in the earlier occurrence of the granulomatous response in the spleen than in the liver.

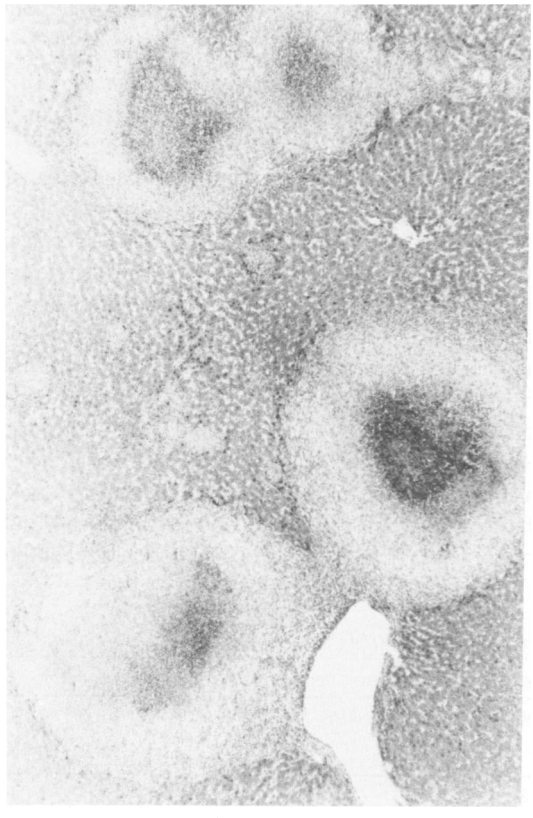

Figure 9. *Multiple hepatic macrogranulomata in vehicle-treated rat 14 days after PG-APS injection. All granulomata shown have central areas of necrosis infiltrated by neutrophils (H&E, ×40).*

Discussion

The findings of this investigation make it possible to set up a definite timetable for the series of events taking place in PG-APS–induced disease in the vehicle-treated Lewis rat (Table 3). All the reactions listed are thought to represent responses to local streptococcal cell wall deposition, which is known to occur within hours after intraperitoneal injection.[12] The actual type of the lesion and its temporal constraints then are determined by the microenvironment.

There was a surprising rapidity to the development and progression of the histologic changes. Thus, acute synovitis was well established after 1 day, the first splenic granulomata appeared after the second day, and hemopoietic hyperplasia and osteoclasia in the tibia became evident after 3 days. A seeming discrepancy, however, existed between the early occurrence of granulomata in the spleen and their manifestation, much later, in the liver. The explanation may lie in a difference in antigen processing in the two locations. The macrophages in the marginal zone of the spleen, a prime antigen trapping site, may be better equipped for attracting monocytes than the Kupffer cells in the hepatic sinusoids. In fact, most of the liver granulomata probably did not develop in association with Kupffer cells, but in relation to PAS-positive macrophages, which made a rather late arrival in the portal areas.

It should be noted again that osteoclasia did not occur by itself, but was always accompanied by hemopoietic hyperplasia. This suggests a common stimulus for both events.

The characteristic grouping of neutrophils with the osteoclasts has not been previously described. It may represent a mechanism for enhancement of bone resorption, in which the neutrophils contribute their degradative enzymes to the lytic process. The term "neutrophil-assisted osteoclasia" seems appropriate for the condition.

At the center of most reactions resulting from PG-APS injection one probably has to place the stimulated monocyte/macrophage and its cytokines. Interleukin-1 has already been shown to be a product of the granulomata and can be held responsible for attracting neutrophils into

the center of the lesions and for promoting fibroblastic proliferation at the periphery.[13] Activated macrophages also have emerged as a controlling force of hemopoiesis, and they were likely involved with causing the medullary hyperplasia in the present experiments. In the mouse at least, bone marrow macrophages have been shown to release erythropoietin, burst-promoting activity, granulocyte macrophage-stimulating factor, and megakaryocyte-stimulating activity.[14-17] Macrophages also can participate in an alternative indirect pathway of hemopoietic regulation. Evidence comes from experiments in which

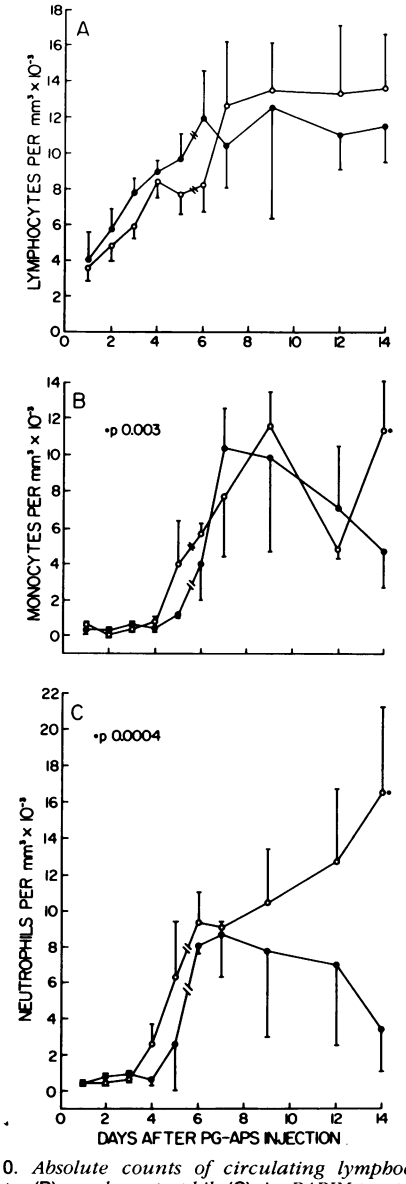

Figure 10. *Absolute counts of circulating lymphocytes* (A), *monocytes* (B), *and neutrophils* (C) *in BABIM-treated (-●-) and vehicle-treated (-○-) rats followed for 2 weeks after PG-APS injection. All groups consisted of four animals, except the day-14 groups which were made up of five animals. Values are means ± SD. Differences between the two series of animals were significant at the points indicated.*

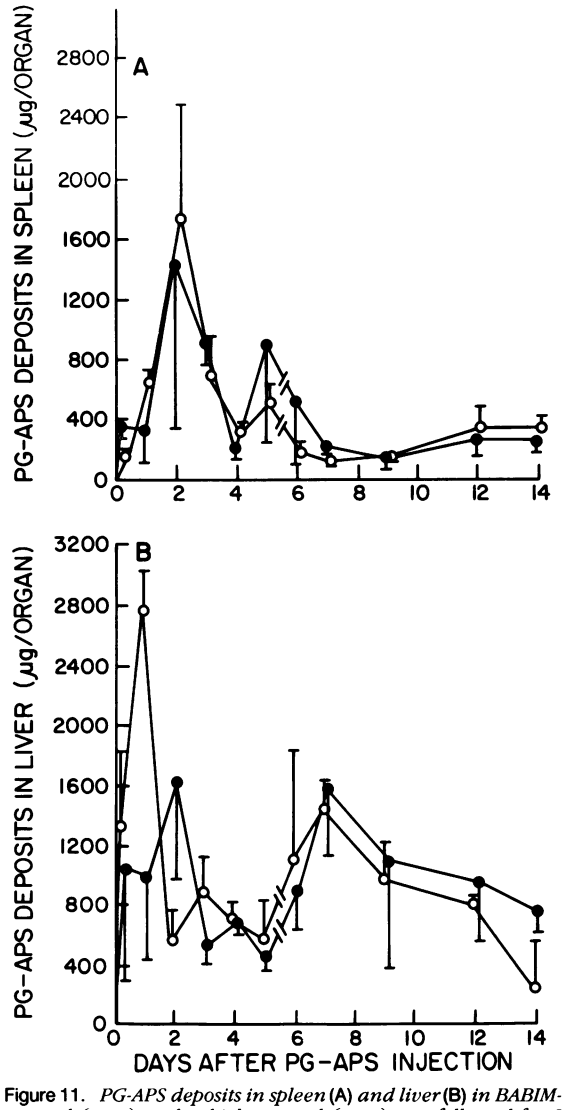

Figure 11. *PG-APS deposits in spleen* (A) *and liver* (B) *in BABIM-treated (-●-) and vehicle-treated (-○-) rats followed for 2 weeks after injection of the streptococcal cell wall material. There were five animals in the day-14 groups. All the other groups consisted of four animals, with the exception of three groups with only three animals and one group with only two animals. Values are means ± SD.*

monokines have been shown to bring about secretion of granulocyte/macrophage growth factor from fibroblasts and erythroid burst-promoting activity from endothelial cells.[18,19] Availability of growth factor probably contributed to the proliferation of osteoclasts in the bone. Osteoclasts are derived from marrow stem cells and share with the other hemopoietic elements the sensitivity to stimulation by interleukin-3.[20,21] Additional factors, such as osteoclast-activating factor and PGE_2, may have further promoted the resorptive activity of the osteoclasts.[22,23]

At this point, it is uncertain what may have caused the mitotic burst in the liver parenchymal cells. Kupffer cells may have been involved, because they are known to react to ingestion of a number of agents by the release of

Table 3. *Chronology of Histologic Responses to PG-APS Injection*

Site	Response	Severity of reaction* Days after injection 1	2	3	4	5	6	7	9	12	14
Synovium	Inflammation	+	++	+++	+++	+++	+++	+++	+++	++++	++++
Tibia	Hemopoiesis	0	0	+	++	++	++++	++++	++++	++++	++++
	Osteoclasia	0	0	+	++	++	++++	++++	++++	++++	++++
	Fibrosis	0	0	0	0	0	0	++	++++	++++	++++
Spleen	Granulomata	0	+	+	++	++	++++	++++	++++	+++	+++
	Perigranulomatous fibrosis	0	0	0	0	0	0	+	++	+++	+++
	Immune response	0	0	0	+	++	+++	+++	+	0	0
	Hemopoiesis	0	0	0	0	0	+	+	+++	++++	++++
Liver	Increased mitotic rate	0	0	0	0	0	++++	++++	++++	0	0
	Microgranulomata	0	0	0	0	0	0	0	+	++	++++
	Macrogranulomata	0	0	0	0	0	0	0	0	+	++

* Intensity of the response is on a scale from 0 to 4+.

IL-1 and of hepatocyte-stimulating factor,[24] and because a similar response may happen after uptake of PG-APS. However, neither of the two factors has yet been shown to increase hepatic cell division, and the reaction time to their presence should have been measurable in hours rather than days.

Prolonged leukocytosis has been recognized as a significant feature of streptococcal cell wall-induced arthritis and is thought to be an indicator of persistent disease.[25] In view of the other beneficial effects of BABIM in the arthritis model, it came as no surprise that the compound would also cause a reduction in circulating neutrophils and possibly in monocytes. It was interesting though, that BABIM did not seem to significantly mitigate neutrophilia during the first week, at a time when it suppressed inflammatory cell infiltration in tissues such as synovium. The discrepancy may simply be a statistical aberration due to small sample size, or it may indicate that different, as yet unidentified factors govern leukocytosis in the two phases of the experiment. Increasing neutrophilia in the untreated animals in the late stage probably was related to the severe granulomatous liver disease evolving at that time. Animals with BABIM treatment had not only much less liver involvement, but also a much lesser degree of neutrophilia.

As was shown above, the beneficial effects of BABIM were noted in the joints and bones as well as in the spleens and livers. The remarkable activity of the compound was not due to interference with the deposition of PG-APS, because similar amounts were measured in livers and spleens of treated and untreated animals. Rather, it seems most plausible that BABIM intervenes in the processing of the streptococcal cell wall fragments in the macrophages or the transmission of signals from those cells. Points of interference may include, for example, the plasmin-induced release of interleukin-1 from activated monocytes[26] and the transmembrane signalling in cytotoxic T cells, which involves a trypsinlike protease.[27] BABIM is unique in its ability to block acute synovitis as well as in the formation of antigen-induced granulomata, and it may represent the prototype for drugs effective in noninfectious granulomatous conditions, such as Crohn's disease and sarcoidosis.

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