# The Relation of Experimental Arthritis to the Distribution of Streptococcal Cell Wall Fragments

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**The intraperitoneal injection of peptidoglycan-carbohydrate fragments from Group A streptococci produces a chronic, polyarticular, erosive synovitis in rats. The cell wall material accumulates rapidly in the liver, spleen, and lymph nodes, where it causes little injury. At the same time, selective localization and persistence of the material in the synovial and periarticular tissues occurs. Its presence in the joint is associated with acute and recurrent inflammation with focal synovitis, pannus formation, joint destruction, and ankylosis. Cell wall fragments become localized in the synovial and periarticular tissues at a time when there are leukocytes in the bloodstream, which appear to contain the material. During this early phase vascular lesions appear in the synovium and in periarticular tissues with collections of fibrin, neutrophils, macrophages, and cell wall fragments near the venules and capillaries. Recurrent episodes of inflammation and joint injury, associated with persistent cell wall antigen within macrophages, were observed over a period of 90 days. (Am J Pathol 1980, 100:383-402)**

RECURRENT, EROSIVE, POLYARTICULAR ARTHRITIS occurs in rats following the intraperitoneal injection of a sterile aqueous suspension of cell wall fragments from sonicated Group A streptococcal cells.[1] Inflammation of the wrists, paws, ankles, and feet of Sprague–Dawley rats begins 1 or 2 days after the injection, reaches a peak during the first week, and often goes into remission only to recur with later episodes of arthritis.[1] The severity of the arthritis varies from one animal to another, but in most rats the episodes of recurrent or persistent arthritis lead to destruction of the distal joints after several months.[2] Cell wall antigen has been found in the joints of these animals up to 26 days after injection [1] and is evidently responsible for the intense inflammatory reaction since injection of the same material directly into the skin or joints of rabbits will also cause severe, recurrent, localized inflammation and injury.[3-5]

Many features of this model of arthritis are similar to those of human rheumatoid arthritis, for example, polyarticular involvement, episodes of reactivation, severe synovitis, pannus, destruction of cartilage, erosion of

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bone, new subperiosteal bone formation, osteoporosis, and ankylosis.[1,2,6] There are, however, differences. The most important morphologic difference is that we find few lymphocytes and no lymph follicles in the later stage of this model,[1] whereas lymphocytes and plasma cells are prominent in human rheumatoid arthritis [7] (Lymph follicles do occur in rabbit joints injected with streptococcal cell wall fragments [5]).

To date, the morphologic studies of this model have largely focused on the joints and paid less attention to the changes in other organs or tissues.[1] This study was undertaken in an effort to follow the distribution of the cell wall fragments and to further characterize the pathogenesis of this experimental model of arthritis. A group of rats was killed, and complete autopsies were performed at predetermined intervals, ranging from 1 hour to 90 days after the intraperitoneal injection of streptococcal cell wall fragments. Histochemistry, immunofluorescence, and electron and light microscopy were used to trace the distribution of cell wall material in various organs and to study the biologic response to it.

## Materials and Methods

### Experimental Animals

Sixty-six male and female, outbred, Sprague–Dawley rats (Zivic-Miller Laboratory, Allison Park, Pa) were used. Each weighed about 150 g at the beginning of the experiment.

### Preparation of Cell Wall Fragments

Group A, Type 3, Strain D-58 streptococci were grown in Todd–Hewitt broth (Difco, Detroit, Mich.). The cells were harvested, washed, and then disrupted in a Braun shaker (Bronwell Scientific Co., Rochester, NY). Cell walls were collected by differential centrifugation, treated with trypsin and ribonuclease, and washed 5 times. We prepared fragments by suspending cell walls in phosphate-buffered saline (PBS), pH 7.2, and sonicating them for 70 minutes in a Branson sonifier (Heat Systems, Ultrasonics, Plainview, NY). They were filtered through a millipore 0.45-$\mu$ filter, which removed the largest cell wall fragments as well as bacterial cells. The rhamnose and nitrogen concentrations were then determined. The details of these procedures have been described.[1,8]

### Experimental Design

Fifty-two rats were given intraperitoneal injections of Group A streptococcal cell wall fragments equal to 60 $\mu$g rhamnose/g body weight. Fourteen controls were given injections of saline. Clinical evaluations of each rat were recorded every day for the first 2 weeks and every third day thereafter. The method used for grading the joints by direct observation has been described.[1,9]

### Histologic Methods

The rats were divided into 9 groups, each consisting of 5 or 6 test animals and 1 or 2 control animals. Using either vapor, the groups were sacrificed at 1, 3, 6, and 16 hours and at 3, 15, 30, 60, and 90 days after the injection. Complete autopsies followed. Light-microscopic sections of tissues, fixed in 10% phosphate-buffered formalin, were prepared from

the lungs, heart, mediastinal lymph nodes, thymus, spleen, liver, kidneys, and both ankles and feet. The feet were decalcified with the use of formic acid sodium citrate solution and sectioned longitudinally. All light-microscopic sections were stained with hematoxylin–eosin (H&E) and periodic acid–Schiff digest (PAS).

From the groups killed at 6 hours, 16 hours, 3 days, and 15 days tissues were also prepared for electron microscopy from 3 rats (2 test, 1 control). Cubes (1 cu mm) of spleen were fixed in cold 1% osmium tetroxide in phosphate buffer, dehydrated, and embedded in Epon 812. The ankles and feet of these 12 rats were removed, the Achilles tendon severed just above the calcaneus, the lateral ligaments cut, and the ankle flexed to expose the synovium of the talotibial joint. The whole foot was then immersed in cold 1.5% glutaraldehyde in a 0.1 M phosphate buffer. It was allowed to fix for several hours. The synovium, with its underlying fat pad, was then cut into strips with the aid of a dissecting microscope, immersed in 1% osmium tetroxide, dehydrated, and embedded on edge in Epon 812. Adjacent swollen tissues were also cut into 1-cu mm cubes, soaked in osmium, and embedded. The remaining tissue of each foot was further fixed for several days in formalin, decalcified, and prepared for light microscopy.

#### Immunofluorescence

Fluorescein-conjugated rabbit globulin specific for Group A streptococci were prepared as previously described.[10,11] Tissues that had been fixed in formalin, decalcified, and embedded in paraffin were sectioned and cleared of paraffin for 4 days in xylol. Next they were rehydrated and stained in a humid chamber for 30 minutes with fluorescein-conjugated rabbit antiserum and washed with PBS. Controls included sections of tissues from rats not given injections, stained as outlined above, sections from test rats stained with fluorescein-conjugated normal rabbit globulin, and sections from test rats stained first with unconjugated Group A-specific rabbit antisera and then with fluorescein-conjugated Group A-specific antisera. In the positive tissue sections, cell wall antigen appeared as large bright apple-green fluorescent particles, and all controls were negative.

#### Radiology

The day before they were killed, the 14 rats in the groups killed at 60 or 90 days were anesthetized with an intramuscular injection of Innovar (McNeil, Fort Washington, Pa), 0.08–0.1 ml/100 g body weight, and immobilized in a supine position. Radiographs were made of their long extremities with the use of Kodak glass projector slide plates and were evaluated by one of us as previously described.[2]

## Results

#### Clinical Observations

There was no immediate reaction to the IP injection of the cell walls. Most rats developed acute arthritis in all four extremities, with accompanying swelling and erythema, the day after injection. This acute phase was greatest on Day 3 in most rats and then subsided, a few rats returning to normal; however, some swelling continued in most rats for the duration of the experiment. The persistent inflammation was aggravated periodically by recurrent episodes of acute arthritis. Some of the rats had only one recurrence; but 4 of the 6 rats observed for 3 months had two distinct episodes. As time passed and the arthritis progressed, the rats eventually

developed enlarged, stiff joints with swelling, osteoporosis, periostitis, erosion, and destruction of cartilage (Figure 1A). Each of these five radiologic features were scored 0 to 4,[2] and the total for each rat was calculated. There was considerable variation from one rat to another, and the radiologic features increased with time. The range of total scores for the rats killed at day 60 was 3–8, and the mean score for the entire group of 5 rats was 5.4. The range for the 5 rats killed at 90 days was 8–13, and the mean score was 10.2. All 4 control rats in both groups were normal (Figure 1B).

#### Morphologic Findings

For the first day after the intraperitoneal injection of the streptococcal cell wall fragments, the rats had a moderate amount of peritoneal exudate that contained many neutrophils. The liver and spleen were covered with a thin acute inflammatory exudate (Figure 2). With time, the reaction subsided, and after 2 weeks the only morphologic change observed was some fibrosis of the capsule of the spleen and liver.

During the first day there was a progressive increase in the amount of PAS-positive material within macrophages of the spleen (Figure 2). Immunofluorescent stains revealed large amounts of streptococcal cell wall antigen within splenic and peritoneal macrophages (Figure 3). Electron microscopy showed that the phagosomes within the macrophages were laden with cell wall fragments that appeared as electron-lucent material (Figure 4). As time progressed, the perisplenitis subsided. The numbers of neutrophils in the spleen also decreased, and the material within the splenic macrophages became more electron-dense. The amount of antigen within the spleen, which immunofluorescence had shown to be greatest at 3 days, slowly decreased and was almost gone by 90 days. There were only rare areas of necrosis in the spleen at any time, and by Day 15 most of the streptococcal antigen remained within splenic macrophages in the red pulp.

By 3 hours small amounts of PAS-positive material could be seen within Kupffer cells throughout the liver. By 16 hours, however, immunofluorescent stains of the liver showed extensive diffuse deposition of streptococcal antigen in almost all Kupffer cells (Figure 5). As time passed, the antigen was concentrated into larger aggregates of macrophages that for the most part lay within the hepatic sinusoids and showed no evidence of acute inflammation. Occasionally one of these aggregates became surrounded by neutrophils (Figure 6), but there was no evidence of hepatic injury. The amount of antigen within the liver was greatest at 3 days. It then slowly diminished and was only seen in trace amounts by 90 days.

The mediastinal tissues contained cell wall fragments 1 hour after the intraperitoneal injection. At first, the PAS-positive material appeared within macrophages in the subcapsular sinusoids of the mediastinal lymph nodes (Figure 7). This acute influx of streptococcal cell wall fragments was associated with a heavy neutrophilic infiltrate, which often extended into adjacent mediastinal fat (Figure 7). Immunofluorescent stains revealed large amounts of streptococcal antigen within the lymph nodes, adjacent fat, and the capsule of the thymus (Figure 8) but never within the thymus itself. By the third day, most of the neutrophils were gone. By the 15th day the acute inflammatory process in the lymph nodes and adjacent tissues had completely subsided, and antigen was deposited within macrophages in the lymph nodes and in the interstitial spaces. Again the amount of antigen in the lymph nodes, which was greatest at Day 3, slowly decreased until only small amounts were present 3 months after injection.

During the first 3 days streptococcal antigen was observed by immunofluorescence to be in bone marrow macrophages. It produced no histologic reaction. Extracellular antigen was observed within pulmonary vessels for the first 16 hours but was gone by 3 days. There were no intravascular thrombi, and no antigen was ever observed within the kidneys or heart. No significant histologic change in the lungs, kidneys, or hearts of any of the rats was detected.

Streptococcal cell wall fragments also accumulated in the ankles and feet of inoculated rats. However, there was less accumulation, and it occurred slightly later in the course of the disease than in the lymph nodes, spleen, and liver. The first histologic changes observed were in the synovial membranes and the subcutaneous tissues of the ankles and feet. By 16 hours there were small perivascular foci of neutrophils and edema in the subcutaneous tissues adjacent to the joints in 3 of 6 rats (Figure 9). Using immunofluorescence, we saw small granules of antigen within cells beside these vessels (Figure 10). Electron microscopy showed neutrophils containing material presumed to be cell wall fragments in the vein wall (Figure 11). The adjacent tissues were swollen and also contained macrophages with small amounts of material resembling the fragments (Figure 12). A similar acute inflammatory reaction was found in some synovial membranes in the ankles and feet of the same 3 rats. Several distended synovial spaces were filled with neutrophils and fibrin (Figure 13). Small amounts of streptococcal antigen were seen near synovial blood vessels by immunofluorescence (not illustrated), and fibrin covered the intact synovium (Figure 14).

By Day 3 the acute inflammatory reaction was most intense, involving all synovial membranes (Figure 15), bursae, muscles, dermis, subcutaneous

tissues, and the periosteum of the ankle and foot. The greatest amount of antigen was seen at this time in macrophages in the skin and subcutaneous tissues around joints, in the adjacent muscles, bursae, synovium, and in the joint spaces. All of these locations were inflamed, and the deposition of antigen appeared directly related to the location and intensity of the acute inflammatory reaction.

By Day 15 most of the inflammation of the subcutaneous tissues and muscles around the ankle joint had subsided. Small deposits of antigen, however, remained within macrophages (Figure 16). Some synovial spaces were distended with a fibrinopurulent exudate, while others were uninvolved. From the 15th day on, antigen could not be demonstrated with certainty within the synovial exudate; yet it persisted for at least 3 months in the synovium and in joint capsules (Figure 17). Cellular pannus was seen in some joint spaces in 2 of 6 rats at Day 15. It consisted of a layer of loose fibrous tissue containing mononuclear cells that covered the cartilaginous surfaces (Figure 18). The pannus did not contain any demonstrable streptococcal antigen.

By day 30 the inflammation of the subcutaneous tissues, muscles, and skin around the joints had completely subsided. Nonetheless, streptococcal cell wall fragments remained in the form of small PAS-positive granules within macrophages. Immunofluorescent stains demonstrated streptococcal antigen (apple green) as well as autofluorescent lipofuscin (yellow green). In contrast, some joint spaces of all 6 rats were severely inflamed, but the involvement was focal, and many synovial spaces appeared uninvolved. Antigen was found in the walls of many synovial membranes with or without inflammation. By now there was definite erosion of the edges of the articular surfaces in inflamed joints, and adjacent subperiosteal new bone began to appear (Figure 19). In some joint spaces the cartilage was partly eroded. There was good correlation between the clinical evaluation and morphology of the arthritis: rats having relapses at the time of sacrifice also had severe inflammation of several synovial spaces. The adjacent muscles and subcutaneous tissues were no longer inflammed.

Five rats were killed at Day 60. One appeared almost normal, and the other four had chronic changes with new bone formation, ankylosis, and focal synovitis. The amount of antigen was less than in previous groups, but small amounts of antigen could still be demonstrated adjacent to joints and in the synovium. Two rats had pannus.

The ankles of the 5 rats sacrificed 90 days after injection were all severely damaged. Each ankle contained one or two distended synovial spaces that were filled with neutrophils (Figure 20), but there were very few lymphocytes and no lymph follicles. The walls of those synovial

spaces contained macrophages laden with lipochrome but very little streptococcal antigen. There was marked osteoporosis and new bone formation. Also, several joint spaces in each foot were destroyed and replaced by fibrous tissue (Figure 21). There was no pannus.

## Discussion

The experiment was designed to study the distribution of streptococcal cell wall fragments and the associated anatomic changes following intraperitoneal injection into Sprague–Dowley rats. The material causes an acute peritonitis within the first 3 hours. It then quickly enters the lymphatics and bloodstream, where it is observed extracellularly and in phagocytes. Most of these cell wall fragments are removed by the reticuloendothelial system in the spleen, liver, and mediastinal lymph nodes. Other lymph nodes were not examined. During the first 3 days, these deposits of cell wall fragments were associated with an intense neutrophilic response; but within 2 weeks, the inflammation subsided and the cell wall fragments were sequestered in macrophages in these organs, where they remained and were slowly eliminated. Occasionally there was a localized recurrence of the neutrophilic attraction, but for the most part, the material caused very little injury. This result was in sharp contrast to what was observed in the joints.

During the first few days after the injection, when there were circulating leukocytes with antigen as well as extracellular antigen, cell wall fragments accumulated within the ankles and feet of all rats. The cell wall fragments first appeared at 16 hours and reached a maximum at 3 days. They were distributed throughout many periarticular foci of the foot and ankle: in the dermis, subcutaneous tissue, and muscles; around tendons; and in bursae, synovial spaces, and synovial membranes. By 3 days all these periarticular tissues were acutely inflamed and many synovial spaces were filled with fibrin, neutrophils, and macrophages. The wrists and paws were also inflamed but were not examined histologically. There is some evidence that the inflammatory reaction itself influences the deposition of cell wall fragments in that cell wall material is seen within leukocytes traversing venules. The early deposition of streptococcal antigen, in areas of the most intense inflammation, further supports this conclusion.

With time, the acute reaction subsided; the cell wall fragments became sequestered in macrophages, some of which are located within synovial membranes. Episodes of acute inflammation recurred within individual synovial spaces, fibrin and neutrophils collected in joints, pannus formed, articular surfaces slowly eroded, and the joints were finally destroyed. The amount of streptococcal antigen slowly decreased from Day 3 and was al-

most gone by Day 90; but the episodes of reactivation were always associated with the local presence of antigen. The intracellular streptococcal cell wall fragments located outside the synovial membranes elicited no inflammatory reaction after Day 15.

Several questions need to be answered before the unique biologic properties of this chemically defined cell wall material will be understood. These include: *a*) What mechanism is responsible for the selective localization of this material outside the RE system in different species of experimental animals [12,13]? *b*) What accounts for a different degree of severity of arthritis in the Buffalo strain of rat [14]? and *c*) What mechanisms of tissue injury are responsible for the initial and recurrent episodes of inflammatory reactions [15,16]?

The studies recorded here suggest that the antigen escapes from the blood stream into the joints and surrounding tissues during the early stages of the experiment in association with the initial acute inflammatory reaction. There is a marked increase in blood vessel permeability, as indicated by accumulation of fibrin and neutrophils at these sites. Whether the cell wall material initiates this inflammatory process at these sites while still in the bloodstream and then leaks from the blood vessels as a result or whether it leaks and then initiates the acute reaction is not clear. It may do both.

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**Figure 1A**—Radiograph of the ankle of a rat 90 days after intraperitoneal inoculation of streptococcal cell wall fragments. There is osteoporosis, subperiosteal new bone, and destruction of some joint spaces. (×4) **B**—Radiograph of control rat, Day 90. There are no changes. (×4)

**Figure 2**—Spleen of rat inoculated intraperitoneally with cell wall fragments 16 hours before death. The capsule is covered by an inflammatory exudate, and the spleen contains neutrophils and PAS-positive macrophages. (PAS, ×330)

**Figure 3**—Spleen from a rat given an intraperitoneal injection of Group A streptococcal cell wall fragments 16 hours before death, stained with fluorescein-conjugated rabbit anti-Group A polysaccharide. Most of the antigen is within macrophages. (×330)


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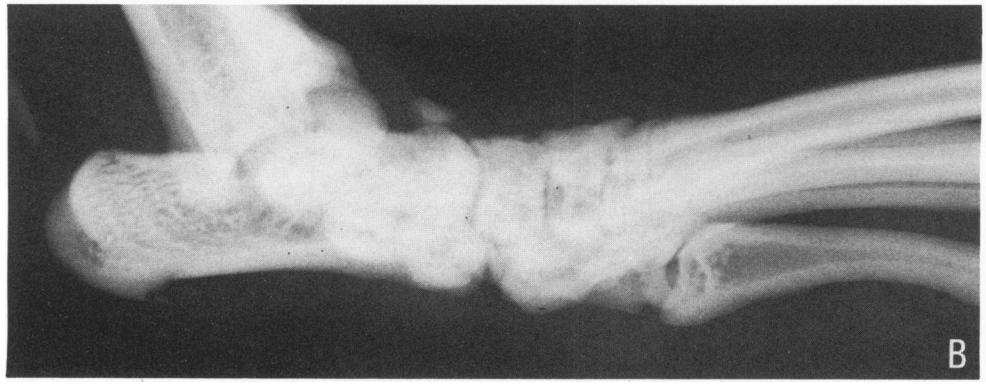


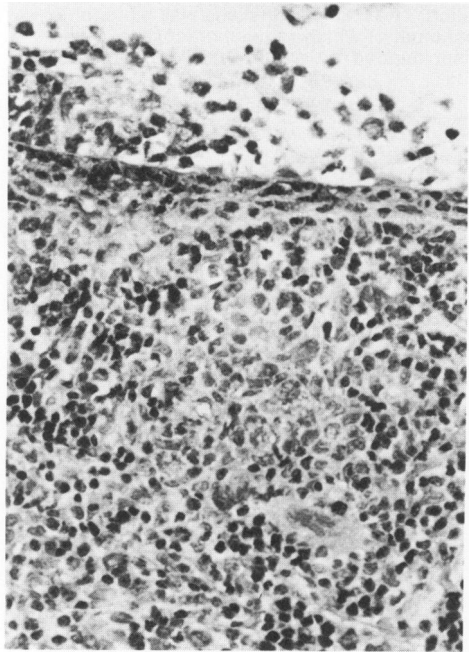

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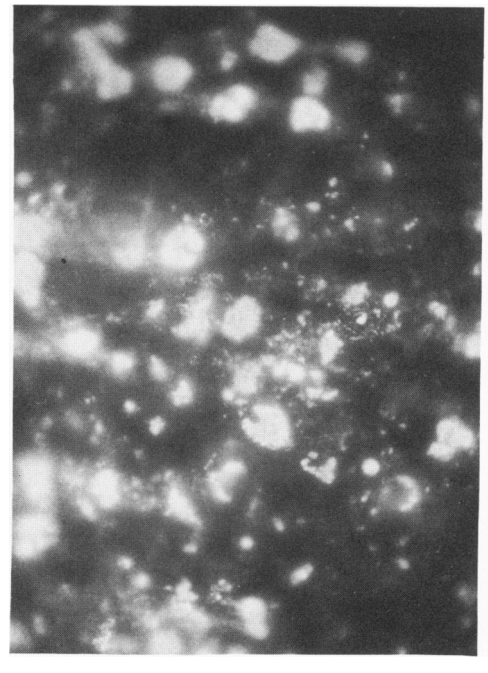

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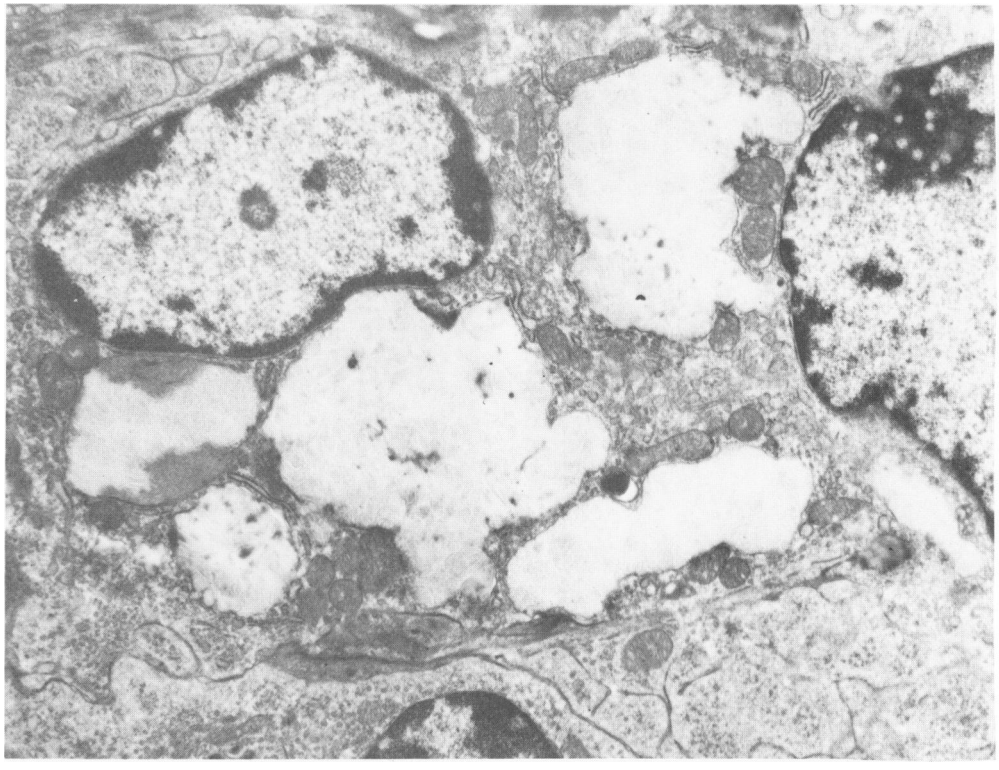
**Figure 4**—Electron micrograph of a macrophage in the spleen of a rat killed 3 days after inoculation. The streptococcal call wall fragments appear as electron-lucent, faintly lamellar material within phagosomes. (×10,000)

**Figure 5**—Liver of a rat killed 16 hours after inoculation, stained with fluorescein-conjugated rabbit anti-Group A polysaccharide. Widespread deposition of streptococcal antigen is apparent. (×85)

**Figure 6**—Liver of a rat killed 15 days after inoculation. The PAS-positive cell wall material is concentrated into large aggregates of macrophages, most of which remain intact (*above* and *below*). Occasional clusters of macrophages are surrounded by neutrophils (*center right*). (PAS, ×330)



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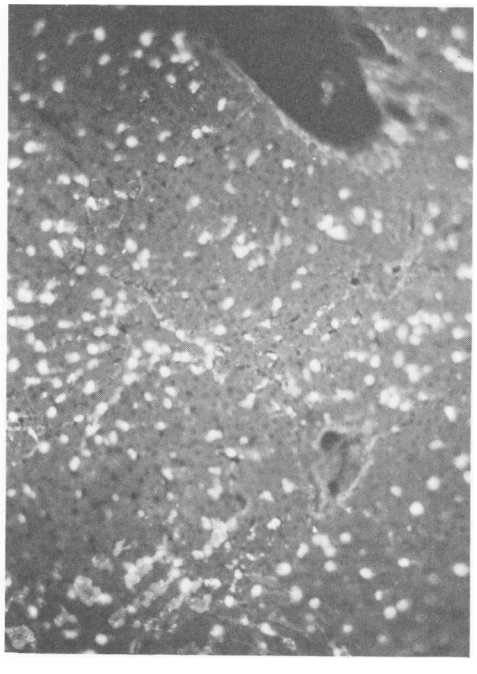

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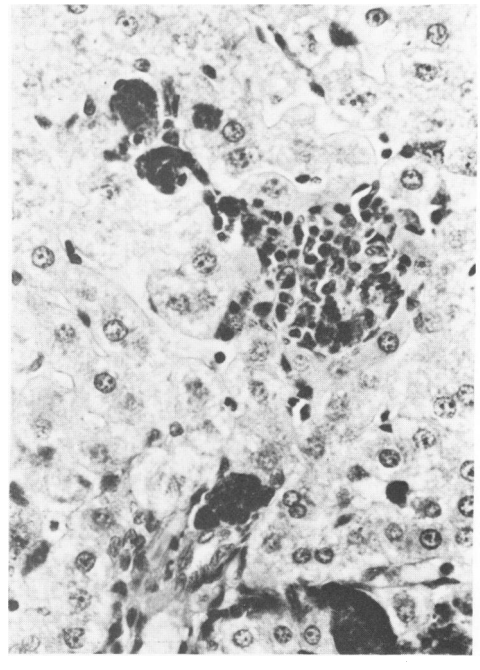

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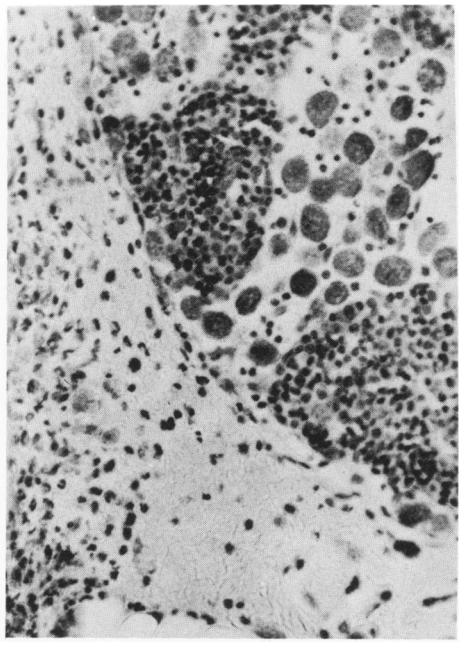

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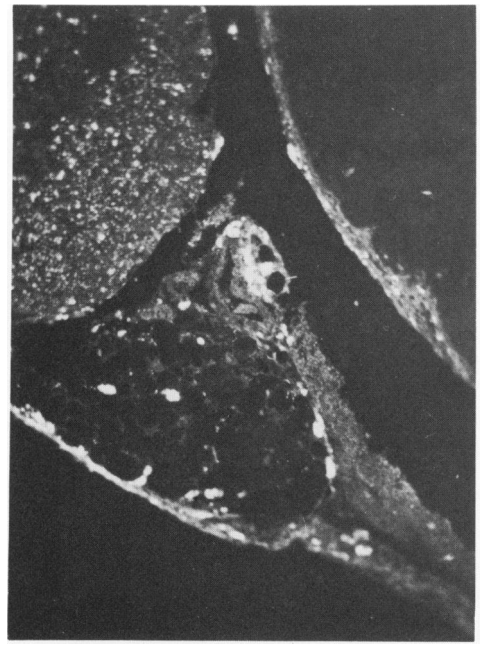

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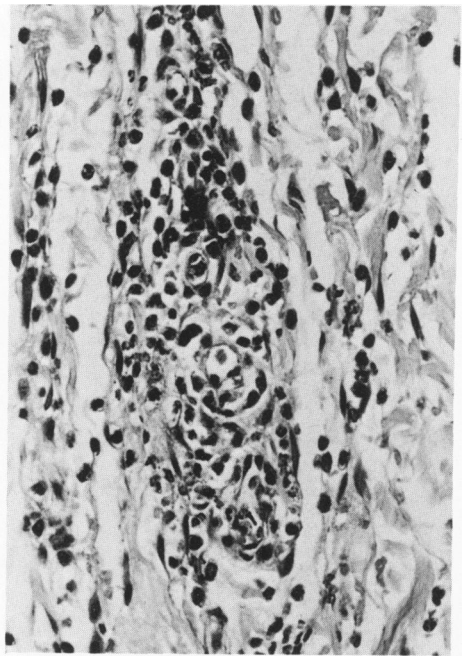

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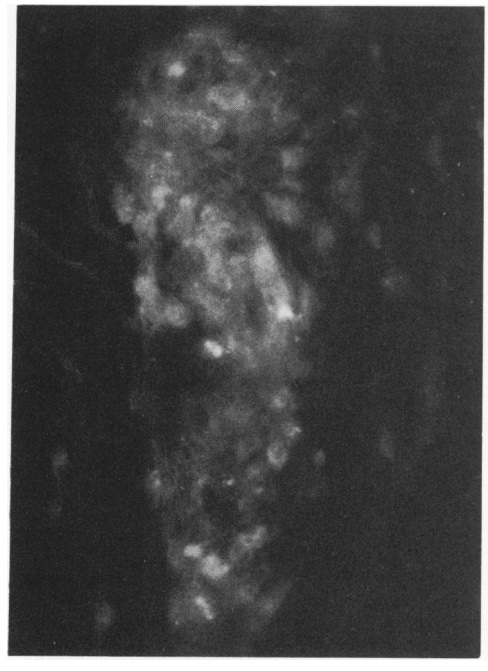

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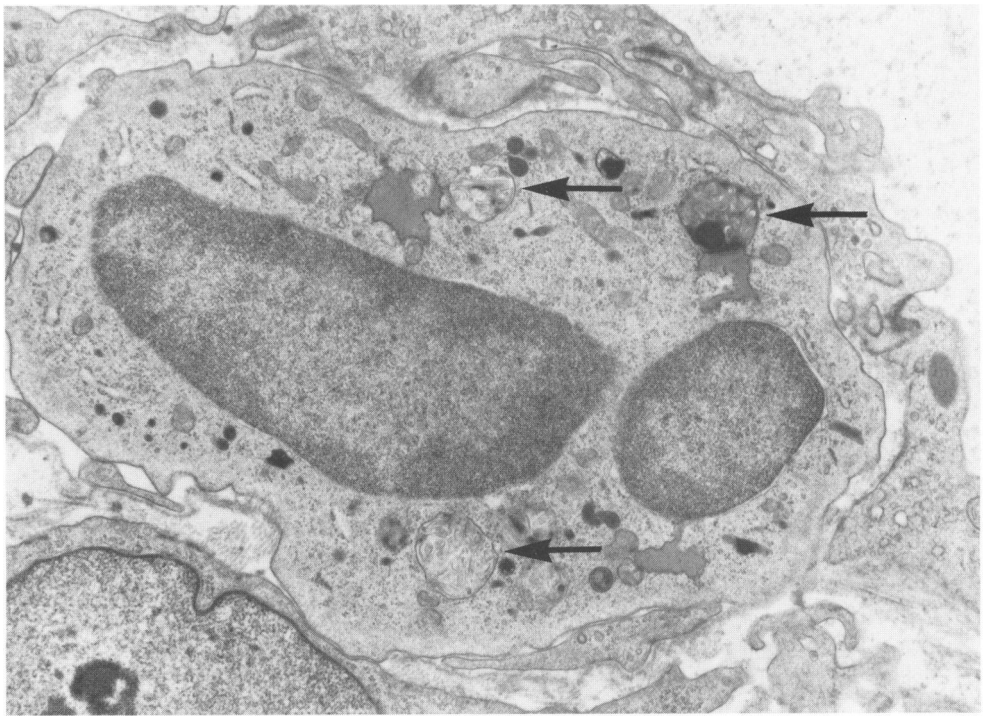
**Figure 7**—Edge of mediastinal lymph node from a rat killed 16 hours after intraperitoneal inoculation. Cell wall has accumulated in macrophages within the node and is also deposited in adjacent tissues, where it has produced an intense neutrophilic response (*lower left*). (PAS, ×285) **Figure 8**—Thymus (*upper right*), lymph node (*upper left*), and adipose tissue (*lower*) from the mediastinum of a rat killed 16 hours after inoculation. Large amounts of streptococcal antigen are located in the lymph node, fat, and capsule of the thymus, but none within the thymus itself. (Immunofluorescent technique, ×82) **Figure 9**—Swollen subcutaneous tissue of the foot of a rat killed 16 hours after inoculation. The small vessel in the center of the field is surrounded by neutrophils. (H&E, ×330) **Figure 10**—Subcutaneous tissue from the same foot illustrated in Figure 9, stained with fluorescein-conjugated anti-Group A polysaccharide. Small granules of streptococcal cell wall antigen are located in perivascular leukocytes. (Immunofluorescent techniques, ×330)



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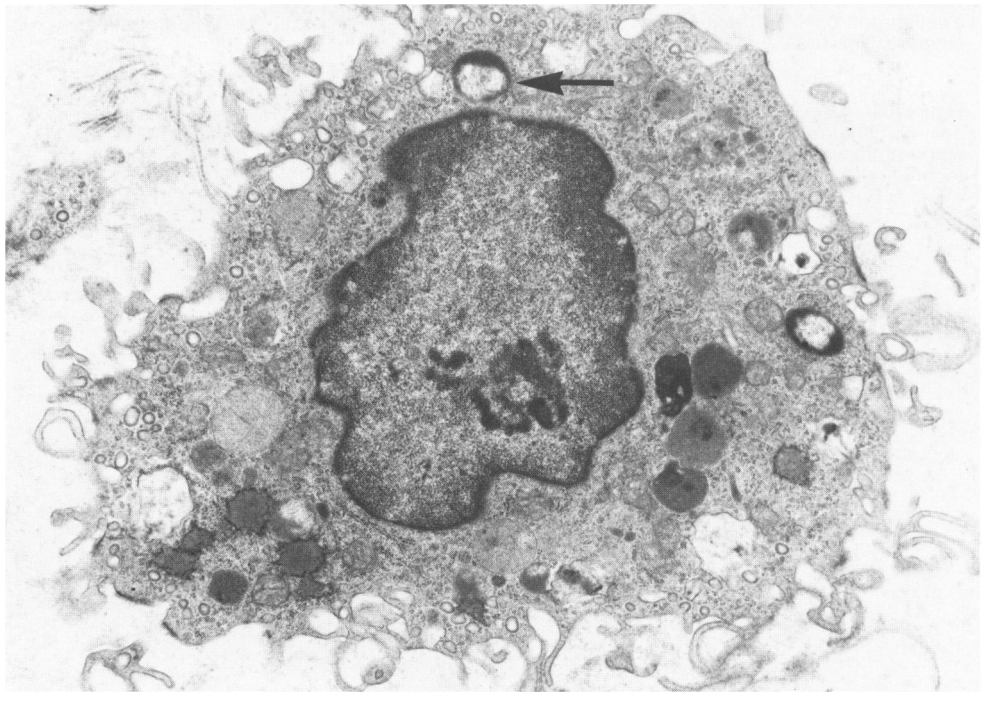

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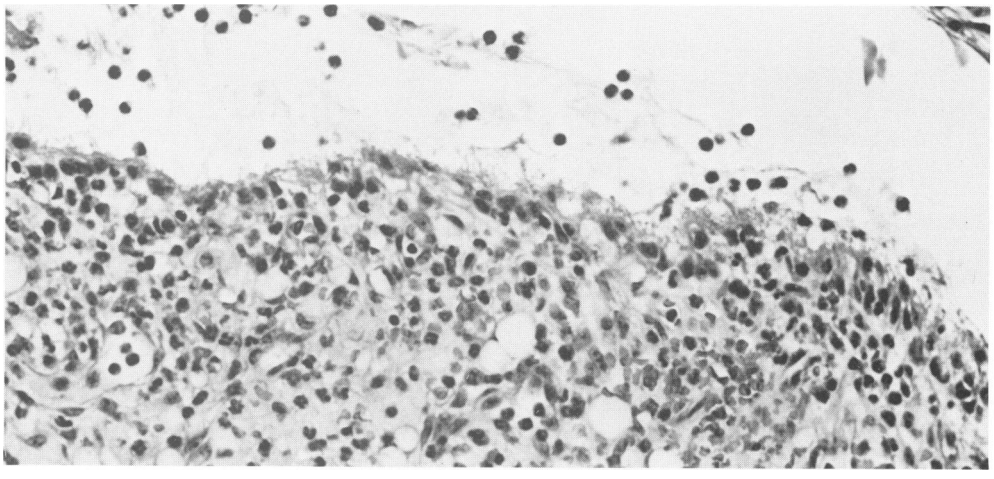
**Figure 11**—Electron micrograph of a vein wall from the foot of a rat killed 16 hours after inoculation. Presumed cell wall material appears within three phagosomes of this degranulated neutrophil (*arrow*). The cell is located just under the intact endothelium and appears to be leaving the vein. (×10,000) **Figure 12**—Electron micrograph of a macrophage in swollen interstitial tissues of a rat foot 16 hours after inoculation. Several small phagosomes contain lamellar material presumed to be cell wall fragments (*arrow*). (×10,000)

**Figure 13**—Synovium from the ankle of a rat killed 16 hours after inoculation. The joint space is distended, and the membrane and fluid contain large numbers of neutrophils. (H&E, ×310)

**Figure 14**—Electron micrograph of the synovium from the foot of a rat killed 16 hours after inoculation. Synovial cells are intact but are covered by an exudate containing fibrin. (×5000)

**Figure 15**—Synovium from the ankle of a rat killed 3 days after inoculation. The membrane is swollen and contains neutrophils and a few macrophages. A large cluster of fibrin is evident (*upper right*). (H&E, ×165)

**Figure 16**—Muscle from the ankle of a rat killed 15 days after inoculation, stained with fluorescein-conjugated antistreptococcal serum. There is no inflammation, but several antigen laden macrophages are located between muscle cells in the middle of the field. (Immunofluorescent technique, ×210)



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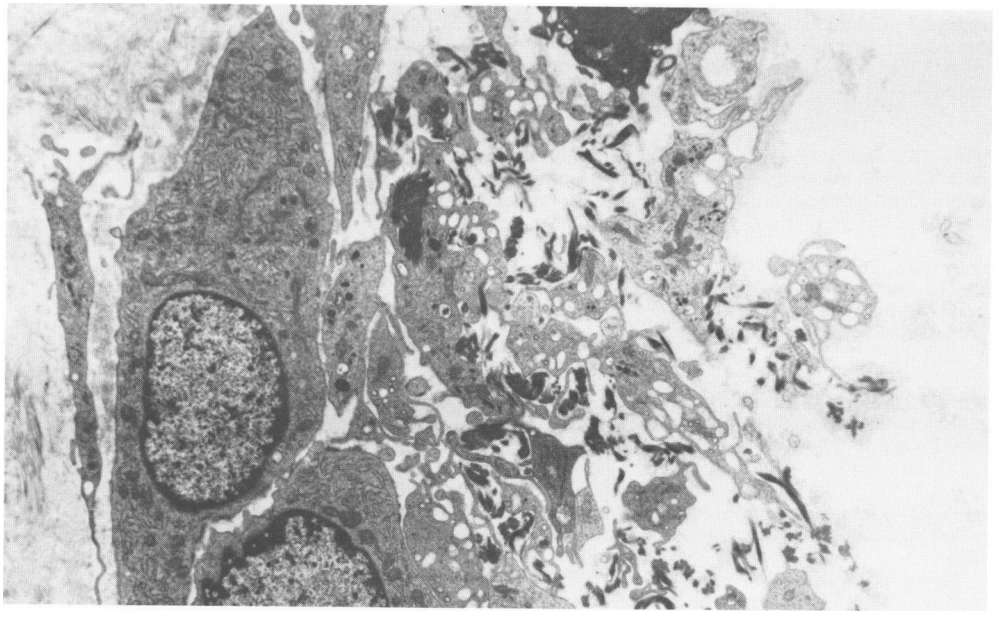

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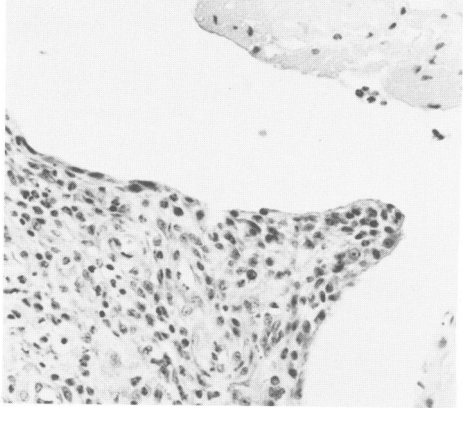

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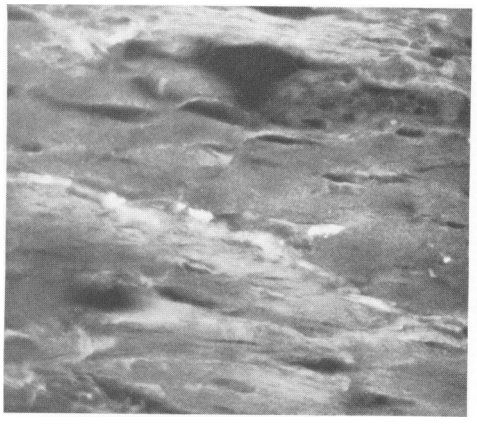

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**Figure 17**—Synovium from the foot of a rat killed 30 days after inoculation. There are several granules of streptococcal antigen with macrophages in the wall of the synovial membrane (*lower*). The synovial cells and joint space do not contain antigen. (Immunofluorescent technique, ×210)

**Figure 18**—Pannus covering the anticular cartilage in ankle joint of rat killed 15 days after inoculation. The pannus is composed of numerous macrophages and some neutrophils in a stroma. The joint space is distended and contains leukocytes and fibrin. (H&E, ×330)

**Figure 19**—The foot of a rat killed 30 days after inoculation. The synovium is thickened, and the joint space is swollen and filled with leukocytes and fibrin. The joint capsule is also thickened, and new bone has formed beneath the periosteum to the left of the joint space. There is some destruction of cartilage. (H&E, ×34)

**Figure 20**—Edge of one synovial membrane from the foot of a rat killed 90 days after inoculation. There were occasional synovial membranes such as this one with thickened inflamed walls and lumens filled with an exudate. (PAS, ×82)

**Figure 21**—Joint space from the foot of the rat killed 90 days after inoculation. The articular surface has been destroyed, and the space has been obliterated by dense fibrous tissue. (PAS, ×82)

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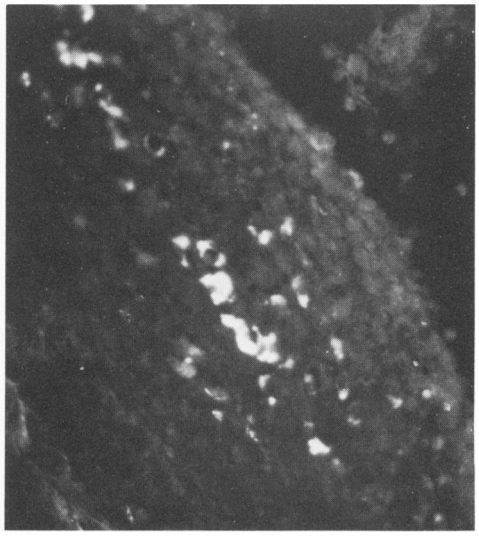

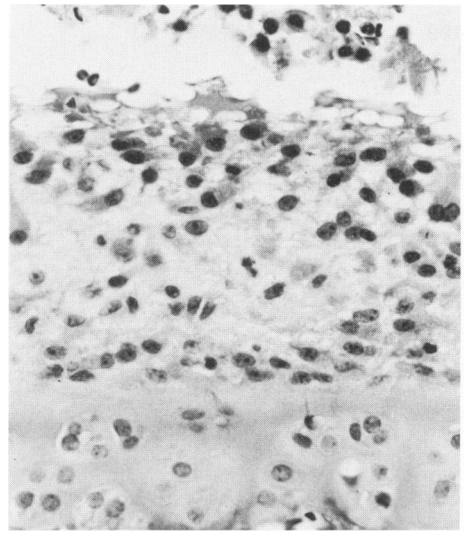

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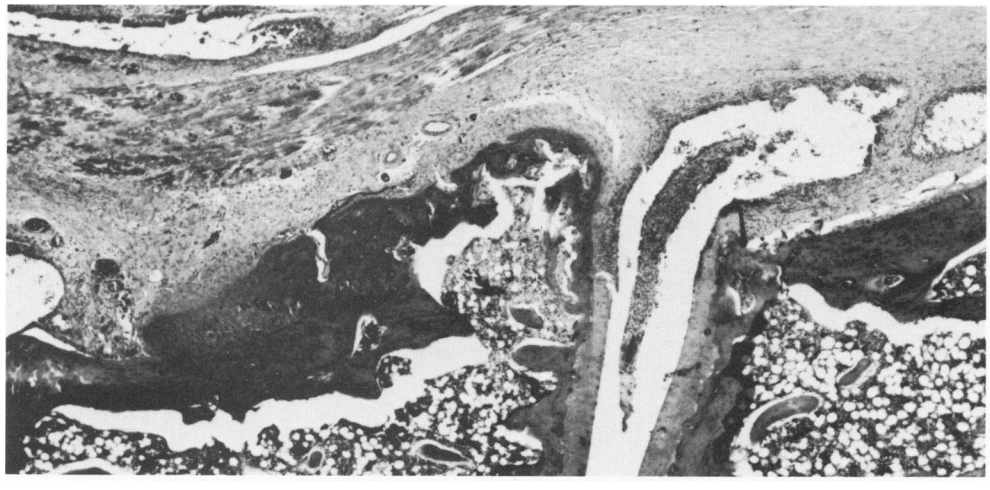

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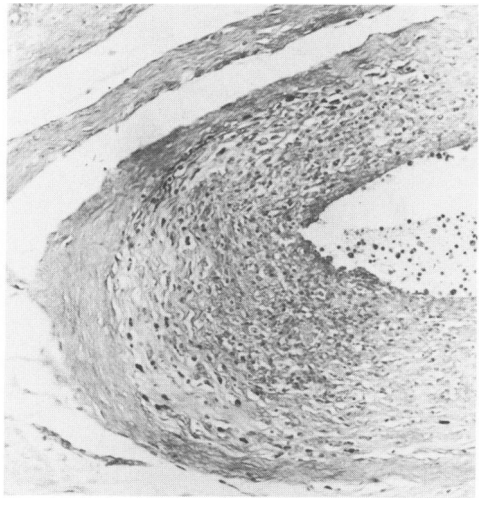

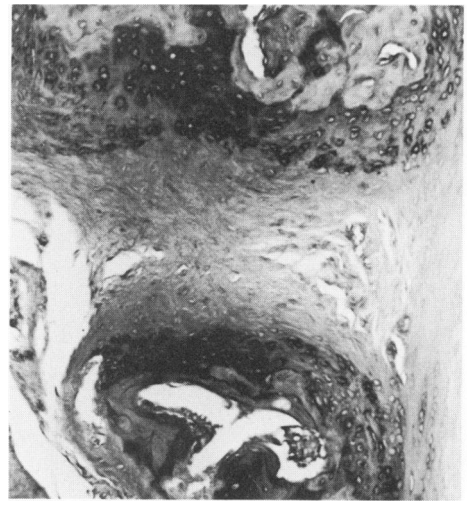

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[*End of Article*]