Immune Synovitis in Rabbits

Effects of Differing Schedules for Intra-Articular Challenge With Antigen

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The effects of varying intra-articular (ia) doses of bovine serum albumin (BSA) antigen on immune synovitis in rabbits have been investigated. Chronic synovitis, characterized by mononuclear cell infiltration in synovial tissues, was induced by a single ia challenge with BSA in sensitized rabbits. However, cartilage and bone erosions and pannus formation were rarely observed. By varying the number and magnitude of the BSA challenges, lesions with different characteristics were observed at different times of analysis of joint pathology. In 3- to 10-week studies, multiple ia challenges with BSA produced lesions characterized by severe cartilage and bone changes; polymorphonuclear leukocyte (PMN) exudates; and mononuclear cells and, sometimes, PMNs in synovial tissues. Substantial increases in knee widths and synovial tissue weights were also observed. By increasing the frequency of ia injections, more severe changes were produced more rapidly, so that within a 3-week period, the animals also experienced pain and were unable to fully extend their antigen-challenged knees. Some of the lesions observed in immune synovitis resembled those in rheumatoid arthritis (RA). However, the presence of large numbers of PMNs in synovial tissue under certain conditions suggests some possible differences between the pathogenesis of experimental synovitis and RA. (Am J Pathol 91:329–344, 1978)

IMMUNE MONO-ARTICULAR SYNOVITIS can be produced in rabbits with a variety of antigens following procedures similar to those described by Dumonde and Glynn.[1] After a single intra-articular (ia) injection of bovine serum albumin (BSA) antigen into sensitized rabbits, an acute Arthus-like inflammation is evident within hours. This reaction consists of an accumulation of polymorphonuclear-leukocyte (PMN)–containing exudative fluid in the joint space, with the synovial tissue containing considerable numbers of PMNs as the major, if not sole, type of infiltrating cell.[2] As this reaction subsides, the chronic stage of synovitis appears and lasts for many months. This stage resembles the histologic picture observed in rheumatoid synovial tissues in that there is marked infiltration of lymphocytes, macrophages, and plasma cells as well as hyperplasia of the synovial tissue.[2-5]

There have been a number of reports describing the effects of multiple ia injections of antigen into rabbits for the purposes of sensitizing and

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subsequently producing chronic synovitis.[cf6-9] In the present report, some qualitiative as well as quantitative aspects of rabbit synovitis have been investigated after ia administration of a soluble, nonimmunoglobulin antigen to sensitized animals using different challenge schedules. Severe lesions were produced that demonstrate exudate and gross pannus formation and cartilage erosions. Milder synovitis was also produced and was characterized by relatively little exudate and minimal or no cartilage and bone changes, with the synovial tissue alone showing signs of inflammation.

Materials and Methods

Sensitization of Rabbits and Induction of Synovitis

Male New Zealand white rabbits, 1.5 to 2 kg, were obtained from a number of local vendors. Animals were allowed water and feed *ad libitum*. Rabbits were sensitized to BSA as previously described.[2] An emulsion was made of equal volumes of BSA (10 mg/ml in pyrogen-free saline [PFS]) and Freund's complete adjuvant. Two milligrams of BSA in adjuvant were injected into the pads of each hind foot. Two to three weeks later, animals were injected intradermally on the back with 250 μg of BSA. The skin responses were usually assessed visually (either + or −) 24 hours later just prior to initiating an experiment. Animals not responding were discarded; this, however, occurred only rarely. Sensitized rabbits were challenged intra-articularly in the shaved right knees with varying amounts of BSA in 0.1 ml PFS; the left knees received PFS alone. To insure sterility after ia injection, all materials were filtered through Swinnex (Millipore, Bedford, Mass.) 0.22-μ esterified cellulose filters prior to infection. Standard microbiologic tests were performed on representative samples of tissues and exudates and indicated a sterile inflammation.

Measurements of Synovitis

Knee Widths

Knee widths were measured with a caliper. Measurements were taken just prior to and at various times after ia challenge with antigen or PFS. The data presented are corrected for zero-time differences in width between right and left knees.

Synovial Tissue Weight

At varying times after ia challenges with BSA, animals were killed by intracardial injections of commercial veterinary euthanasia preparations. The entire soft synovial tissue was excised, patted dry on absorbent paper, and weighed.

Histologic Evaluations

Synovial tissues were fixed in 10% formalin, and slides for histologic evaluation were prepared. Five- to six-μ sections through the synovial membrane and underlying tissue were stained with hematoxylin and eosin. At least two sections per joint were evaluated. These sections were scanned for infiltrating cell types (PMNs, lymphocytes, macrophages, plasma cells) which were then subjectively graded on a 0 to 4 scale, reflecting the overall assessment of the extent of cellular infiltration.[2] Saline-injected knees virtually always gave scores of 0. After removing the synovial tissue capsules, bone and cartilage samples with some attached synovium were taken and fixed in 10% formalin. These tissues were then

decalcified, processed, and stained with hematoxylin and eosin. From a review of longitudinal sections (7 to 8 μ) through both tibia and femur, pannus formation, articular cartilage erosions, marrow involvement, and periosteal inflammation were each scored using an overall assessment as follows: 1 = very slight or very small, 2 = slight or small, 3 = moderate, 4 = marked.

Exudate Analysis

Joint fluids, sometimes extremely thick and dense with cells and debris, were removed with a disposable glass pipette or spatula and placed in tubes at 0 C. The joint space was then flushed with 0.5 ml of cold PFS dropwise with continuous aspiration of the fluid with a disposable pipette. The wash was added to the original exudate. Samples were then stored at −75 C for less than 1 week before evaluation. To disrupt cells in preparation for the β-glucuronidase assay (discussed later), Triton X-100 was added in a small volume (1:10 v/v) to the thawed samples of exudate flushes to give a final concentration of 0.2%. Additional details have been published.[2]

Samples of 0.1 or 0.2 ml were analyzed for β-glucuronidase activity using phenolphthalein glucuronide as substrate.[2] The optical densities at 550 nm of the appropriately treated supernatants were recorded after an 18-hour incubation. These optical density values were then converted into total numbers of PMN equivalents per joint as previously described.[2]

Miscellaneous Materials

Isotonic PFS was purchased from Abbott Laboratories (North Chicago, Ill.). Freund's complete adjuvant containing *Mycobacterium butyricum* was purchased from Difco (Detroit). Bovine albumin, crystalline (BSA), was obtained from ICN Pharmaceuticals, Inc. (Cleveland); and Triton X-100 was obtained from Schwarz/Mann (Orangeburg, N.Y.).

Statistical Evaluation of Data

Significance was calculated using the Wilcoxon ranking test utilizing the table in Dixon and Massey's text.[10] An experimental result was considered significant if the P value was less than or equal to 0.05.

Results

Immune synovitis was previously studied after induction with a single ia injection of 1 mg BSA antigen into sensitized rabbits.[2] By 1 month, there were few PMNs, while lymphocytes, macrophages, and plasma cells were characteristic in the synovial tissue. The maximum amount of exudate occurred at 24 hours and declined until it was no longer prominent by 2 months.

A synovitis characterized by pannus formation and gross cartilage and bone erosions was not previously demonstrated utilizing a single ia BSA injection and evaluation over a 2-month period.[2] Therefore, the following protocols were implemented in an attempt to produce these effects.

Ten-Week Study

Animals received either a single ia challenge on Day 0 or multiple ia challenges of BSA (1 mg on Day 0 and 0.5 mg in each subsequent injection) at 2-week intervals for as long as 56 days after the initial ia

injection. Groups of rabbits were killed either 24 hours or 2 weeks after the last ia injection. Exudate formation, histology of the synovial tissues, as well as the extent of cartilage and bone destruction in joints were examined. The results are summarized in Table 1.

The groups killed 24 hours after a BSA injection (Groups 1 and 3 in Table 1) showed large numbers of exudate PMNs and PMNs and mononuclear cells in the synovial tissues. This is exemplified in Figure 1 and is contrasted with the normal synovium in Figure 2. The animals in these groups also demonstrated significant BSA-induced increases in synovial tissue weight. Rabbits receiving multiple ia challenges and killed 2 weeks after the last challenge (Group 5) demonstrated increased tissue weights and an average 6-mm increase in knee width, or more than a 10-fold increase over control joints. Significant PMN exudation was observed. However, predominantly mononuclear cell infiltration was present in the synovial tissues. On the other hand, rabbits killed 15, 57, or 70 days after a single ia challenge (Groups 2, 4, and 6) showed decreased PMN exudates; smaller, relatively insignificant, increases in tissue weights; and predominantly mononuclear cell types in the synovial tissues. Also, significant fibrosis occurred in synovial tissues of animals, regardless of the

Table 1—Ten-Week Study of the Effects of Multiple Intra-Articular BSA Challenges

Group	Day of ia BSA*	Day of sacrifice	Average BSA-induced increase†		Average synovium histology scores‡			
			Exudate (PMN equivalents/ml)	Synovial capsule (mg)	PMNs	Lympho-cytes	Mono-cytes	Plasma cells
1	0,14	15	3.7×10^7	404	3.9§	2.4	1.9	2.1
2	0	15	9.5×10^5	26	0.7	2.6	1.7	2.1
3	0,14 28,42 56	57	1.1×10^8	553	3.1§	3.0	2.6	2.9
4	0	57	4.0×10^6	34	0.1	2.0	2.3	3.3
5	0,14 28,42 56	70	4.8×10^7	367	0.7	2.2	2.5	3.4
6	0	70	4.7×10^5	105	0.0	1.7	1.7	3.1

* Seven rabbits per group received 1 mg BSA on Day 0, and, where applicable, 0.5 mg in each subsequent injection.

† P values for each of the following group comparisons for exudates were ≤ 0.007: 1–2; 3–4; 5–6; 3–5; 4–6. For tissue weights, $P \leq 0.006$ for comparisons between groups 1–2; 3–4; 5–6. $P > 0.05$ for comparisons between Groups 2, 4, and 6; and 1, 3, and 5. PMN equivalents and tissue weights in BSA-challenged knees were corrected for those values in saline-injected knees.

‡ 0 to 4 scale for all cell types. Scores were 0, but rarely 0.5, for control knees.

§ P values when compared separately with Groups 2, 4, 5, and 6 were ≤ 0.001.

ia = intra-articular; BSA = bovine serum albumin.

number of BSA challenges, when killed on Day 57 or 70 (Groups 3 through 6). Cartilage erosions were also noted on gross observations of rabbits receiving multiple antigen challenges but were not observed following a single joint challenge.

More detailed histopathologic examinations were made of both knee joints of 11 rabbits (Groups 5 and 6 in Table 1). The animal identification and microscopic observations are listed in Table 2. Extensive proliferative inflammation of the synovial tissues and involvement of the articular cartilages were observed within the BSA-challenged knee joints of Rabbits 16, 18, 19, 20, and 39. The synovial membranes were greatly thickened due to accumulations of large numbers of inflammatory cells, as suggested in Table 1, and, possibly, proliferation of the synovial tissues. Other microscopic changes in these joints included focal or diffuse erosion and necrosis of the articular cartilage. Frequently, cellular pannus formations were seen on the surface of the affected articular cartilages. In some cases the inflammatory process had extended through the articular cartilage and epiphyseal bone into the adjacent bone marrow. In Rabbits 18, 19, and 39 this inflammatory reaction extended into the adjacent muscular tissues and down along the periosteal surfaces.

Table 2—Detailed Histopathologic Evaluation of Selected Tissues From the Ten-Week Study: BSA-Challenged Joints

	Histologic parameter*				
	Inflammation of synovial tissues	Pannus	Erosion of articular cartilage	Marrow involvement	Periosteal inflammation
Five ia injections (Group 5)†					
Rabbit 16	4	2	3	3	
Rabbit 17					
Rabbit 18	4	3	3	3	3
Rabbit 19	4	2	2	2	3
Rabbit 20	4	2	2		
One ia injection (Group 6)†					
Rabbit 36	2				
Rabbit 38	3				
Rabbit 39	4	4	4	3	3
Rabbit 40					
Rabbit 41	2				
Rabbit 42					

* Scoring: 1 = very slight or very small; 2 = slight or small; 3 = moderate; 4 = marked. Where no indication is given, no remarkable changes were observed.

The saline control knees showed no remarkable changes except for Rabbits 39 and 40, in which a rating of 1 was given for synovial tissue inflammation.

† See Table 1.

ia = intra-articular.

Lesser joint involvement was observed in the BSA-challenged knee joints of Rabbits 36, 38, and 41 (Table 2) and consisted of slight or moderate proliferative inflammation of the synovial membranes. Lesions of articular cartilage were not seen in the sections from these rabbits. No remarkable changes were noted in the small amount of synovial tissue adherent to bone samples from antigen-challenged knees of Rabbits 40 and 42. In a separate analysis of the bulk of the tissue from these animals, significant cellular infiltration (scores of 1 to 3) was observed, as suggested by the average group values in Table 1.

In summary, 4 of 5 animals demonstrated considerable involvement of the joint connective tissues after multiple antigen challenges, while only 1 of 6 in the singly challenged group of animals responded with such severe changes when examined at 70 days.

Three-Week Study

The results of administering three ia injections of BSA per week for 3 weeks are indicated in Table 3. The first injection of BSA contained 1 mg; each subsequent injection of 0.5 mg was administered approximately every other day. Animals were killed 24 hours after the last ia challenge. As indicated in Table 3, there were substantial increases in weights of synovial tissues and in knee widths of multiply challenged animals compared with singly challenged animals. In this experiment, we observed the highest levels of PMNs in exudates of any studies to date. Of significance was the observation that 20 to 40% of the cells in the smears of exudates of all 4 multiply challenged animals examined were cells other than PMNs. This was notable since our previous observations with single ia BSA challenges indicated that non-PMN cells were rare in exudates.

Table 3—Three-Week Study of the Effects of Multiple Intra-Articular BSA Challenges

Group	No. ia BSA doses*	No. animals with gross erosions/ Total No.	Average BSA-induced increase†			Average synovium histology scores†			
			Knee diameter (mm)	Synovial tissue wt (mg)	Exudate (PMN equivalents/ joint)	PMNs	Lym-pho-cytes	Mono-cytes	Plasma cells
1	1	0/8	0.8	120	2.2×10^6	0.7	2.0	1.0	1.9
2	9	7/9	5.9	910	1.6×10^8	3.1	3.1	2.7	2.9

* The first BSA challenge was 1.0 mg; subsequent injections were with 0.5 mg (total of three injections/week). Animals were killed on Day 22.

† All measurements of BSA-challenged knees at time of sacrifice were corrected for those values of saline-injected knees. Histology scores were 0 for all saline-injected knees. P values for comparisons between Groups 1 and 2 for all measurements were < 0.001, except for plasma cells ($P = 0.01$).

ia = intra-articular; BSA = bovine serum albumin.

Histologically, the synovial tissues from multiply challenged rabbits were infiltrated by all cell types, including PMNs. In addition, a high incidence of cartilage erosions was noted on gross observation. Tissues from those animals receiving only one ia injection of BSA showed less intense infiltrates of all cell types, PMNs being the least prominent. None of the 8 animals receiving a single ia challenge of BSA showed erosions, while 7 of 9 multiply challenged animals did. This experiment also marked the first time that we observed decreased joint function, as evidenced by difficulty in extending BSA-challenged knees. The animals obviously experienced pain on forced movement of these BSA-challenged knees.

Multiple, Reduced Intra-Articular Doses of BSA: Three-and-One-Half-Week Study

In the final study, the number and magnitude of ia doses of BSA were decreased to a point where bone and cartilage pathology could just be demonstrated by histologic examination. Intra-articular BSA doses ranged from 0.02 to 0.5 mg. Animals were evaluated on Day 24. Antigen-induced increases in knee widths were minimal, which suggested a considerably milder synovitis than that previously observed (Table 3). Measurement of PMN levels in exudates showed large intragroup variations that made it impossible to conclude significance. The results of histologic evaluation of synovial tissues from BSA-challenged knees are indicated in Table 4. The synovial tissues of all animals in this study demonstrated significant in-

Table 4—Effects of Multiple, Reduced Intra-Articular Doses of BSA

		ia BSA		Average synovium histology scores†			
Group*	No. rabbits	Dose (mg)	Day	PMNs	Lympho-cytes	Mono-cytes	Plasma cells
1	5	0.5 0.1	0 3,7	0.5	2.8	1.8	2.6
2	4	0.5 0.1	0 5	0	2.5	1.5	2.3
3	4	0.1	0,5	0	2.5	1.8	2.0
4	4	0.1 0.02	0 3,5,7	0	2.0	1.5	2.0
5	5	0.1 0.02	0 5	0	1.6	1.2	1.4

* All groups were killed on Day 24.
† BSA-challenged knees; scores graded 0 to 4; scores were 0 for control knees.
ia = intra-articular; BSA = bovine serum albumin.

flammatory cell infiltration consisting of lymphocytes, monocytes or macrophages, and plasma cells. Virtually no PMNs were observed. Saline-treated knees were histologically normal. There was a tendency for the tissue histology scores of Group 5 to be lower than those for the other groups; significance levels (P) between 0.03 and 0.08 were observed for the various cell types when compared with Group 1. The right knee joints of 2 of the 4 rabbits evaluated in Group 1 (Table 4) showed small areas of erosion and pannus formation (Figure 3) on the articular cartilages. This type of joint pathology was not observed in the other 2 animals of this group. For comparison, a histologic section of cartilage from a normal saline-treated knee is indicated in Figure 4. Articular erosions or pannus formations were not observed in the BSA-challenged knees of any of the rabbits in Groups 2 and 4 (Table 4). Knees from rabbits of Groups 3 and 5 were not examined histologically for cartilage and bone changes.

From the studies presented, it appears that with the level of sensitization obtained in our rabbits, an initial ia challenge with 0.5 mg of BSA followed by two 0.1-mg doses (Group 1 in Table 4) will produce cartilage erosions on histologic examination in some animals within 24 days. However, even with the lowest ia doses used (Group 5 in Table 4), mononuclear cell infiltrates were still observed in synovial tissue after 24 days.

Discussion

Previous work from our laboratory primarily concerned the acute Arthus phase of immune synovitis in rabbits.[2] However, some data were presented that demonstrated that the synovitis becomes chronic (mononuclear cell infiltration) after the initial peak of PMN infiltration in the synovial tissue following a single ia BSA antigen challenge. By 1 month, relatively few PMNs were present in synovial tissues. We observed no gross erosions of articular cartilage up to 2 months after joint challenge. In contrast, Dumonde and Glynn [1] demonstrated pannus formation and cartilage erosions within 2 months after a single large ia dose of autologous or heterologous fibrin.

The use of protocols involving multiple ia antigen challenges into normal or sensitized rabbits has been reported.[6-9] In the present studies, it has been demonstrated that by varying the number and magnitude of ia doses of a soluble, nonimmunoglobulin antigen, ie, BSA, it was possible to produce certain pathologic effects not previously observed in our laboratory utilizing a single ia BSA challenge.[2] Following multiple ia injections of this antigen, gross and histologic examinations indicated that cartilage and bone erosions and pannus formations occurred during a 3- to 10-week period in three different studies (Tables 1 through 4). Using biochemical

assays, we have observed that proteoglycan depletion from cartilage occurs.[11] By decreasing the number of ia injections and the amounts of BSA used, destruction of cartilage was diminished, while mononuclear cell infiltration still was evident in the synovial tissues (Table 4).

Certain patterns of pathologic changes were observed during the evolution of synovitis and are of interest. By varying the protocols, it was possible to demonstrate lesions characterized by PMN-containing exudates and synovial tissue infiltrated with mononuclear cells as well as PMNs (Groups 1 and 3 in Table 1). However, if the time of sacrifice of animals was 2 weeks after the last ia challenge with antigen, eg, Group 5 in Table 1, the lesions were defined by the presence of exudate but with drastically reduced numbers of PMNs in synovial tissues. Mononuclear cells were heavily infiltrated in these tissues. This latter observation is more characteristic of rheumatoid tissue in that many rheumatoid patients have copious amounts of joint exudate with high levels of PMNs, although their synovial tissues on histologic evaluation do not contain a prominent PMN infiltrate.[5] It might be expected that PMNs should be heavily infiltrated in the synovial tissues in some rheumatoid patients at least within hours after an acute flare-up occurs. The lack of documentation of substantial numbers of PMNs in tissues may reflect the time that biopsies are usually taken or the possible differences between humans and rabbits in the pathogenesis and persistence of the synovitis. There are, however, reports [see 5] suggesting that PMNs may be more prevalent in synovial tissues from early rheumatoid cases compared with long-standing RA. This clinical situation may be reflected in the rabbit model presently under investigation. Finally, our data indicate that the most severe joint destruction occurs in the presence of large PMN exudates in the joint whether or not PMNs are prevalent in the synovial tissue at the time of death (Table 1, Groups 3 and 5; Table 3). From this data we cannot draw a causal relationship between PMNs and joint destruction, but these observations may be of relevance in determining the relative importance of PMNs and mononuclear cells in tissue breakdown.

Other investigators have studied possible factors involved in the chronicity of this experimental synovitis. These factors include immunoglobulin synthesis by synovial tissue,[5,12] deposition of immune complexes and complement in cartilage,[13,14] and the release of lymphokines from synovial tissue.[15–17] A number of published reports [cf 18–20] suggest that the ability to produce a delayed hypersensitivity response (such as by use of complete Freund's adjuvant [FCA] in sensitization procedures) is a necessary condition for the production of chronic synovitis in rabbits. However, if FCA-treated animals can produce autoimmune responses, this could be an

alternative or additional factor in eliciting a chronic response.[19] Additional studies on this chronic synovitis model may clarify the mechanisms involved in perpetuation of RA and may suggest new pharmacologic approaches for the development of more efficacious antirheumatic drugs.

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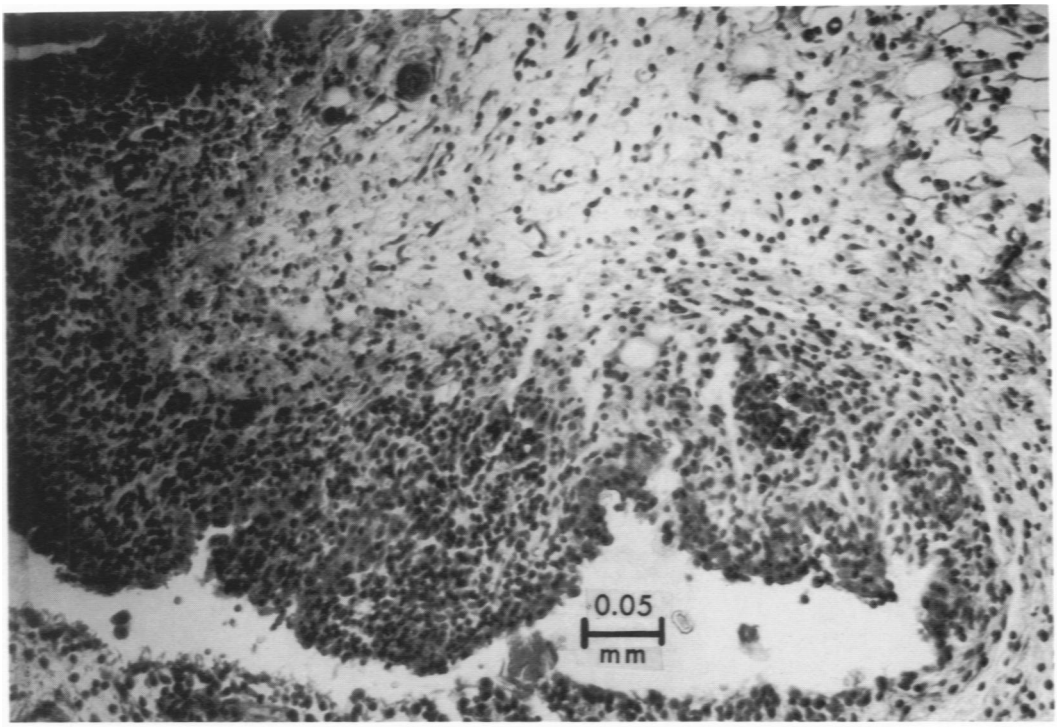
[*Illustrations follow*]



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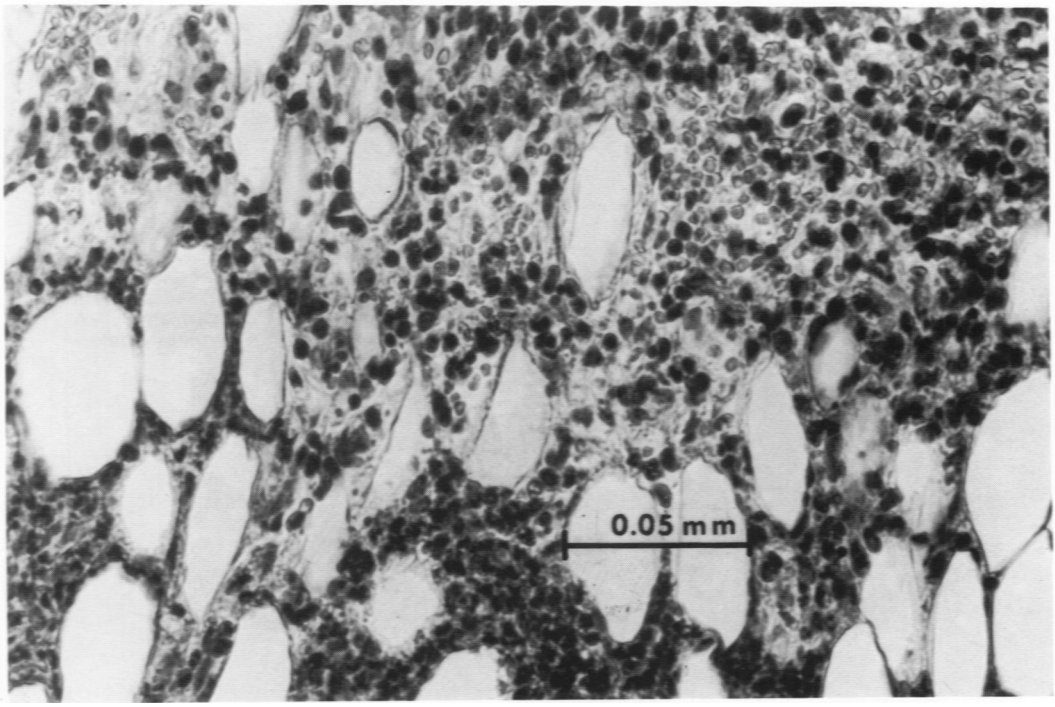
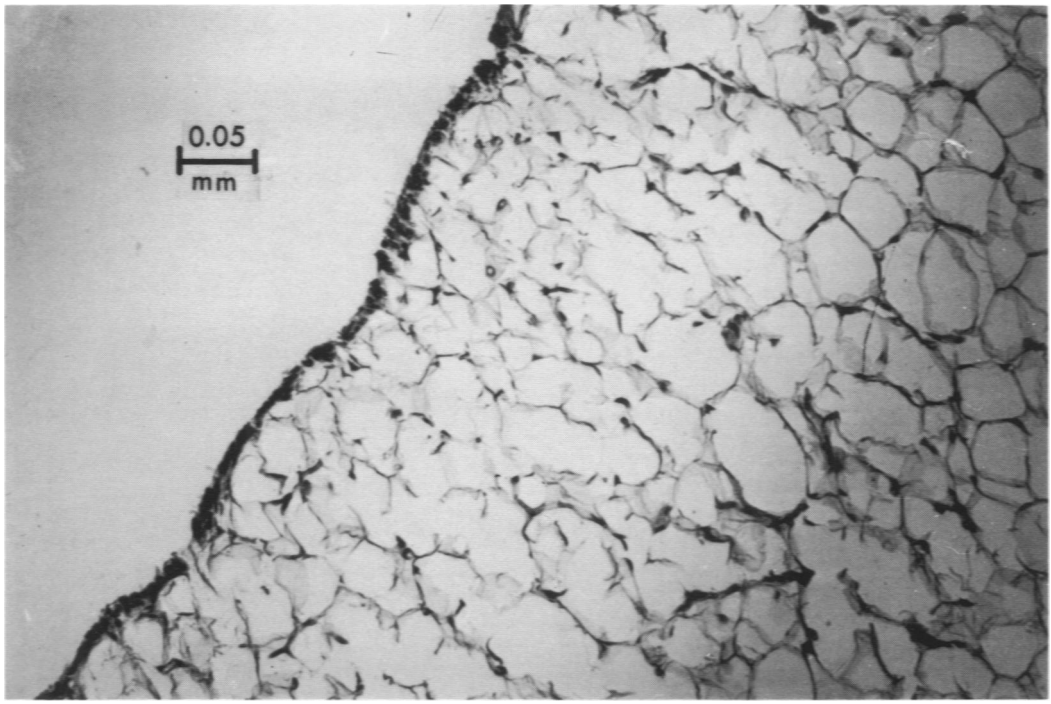


1B

Figure 1—A histologic section of synovial tissue from a rabbit's knee that was injected with 1 mg bovine serum albumin on Day 0 and with 0.5 mg on Day 14. The tissue was removed 24 hours after the second injection. Heavy infiltration by polymorphonuclear leukocytes, lymphocytes, macrophages, and plasma cells can be seen. (H&E; **A**, × 140; **B**, × 350)

Figure 2—Normal rabbit synovial tissue. No cellular infiltrate is observed, and the synovial lining shows no hypertrophy or hyperplasia. (H&E, × 140)

Figure 3—A histologic section of cartilage from the femur of a rabbit that received intra-articular challenges of 0.5 mg BSA on Day 0 and of 0.1 mg on Days 3 and 7. The tissue was obtained on Day 24. Pannus (*P*) is evident overlying the remnants of articular cartilage (*C*). A cellular infiltrate in the pannus can be seen although cell types could not be clearly determined. (H&E, × 140)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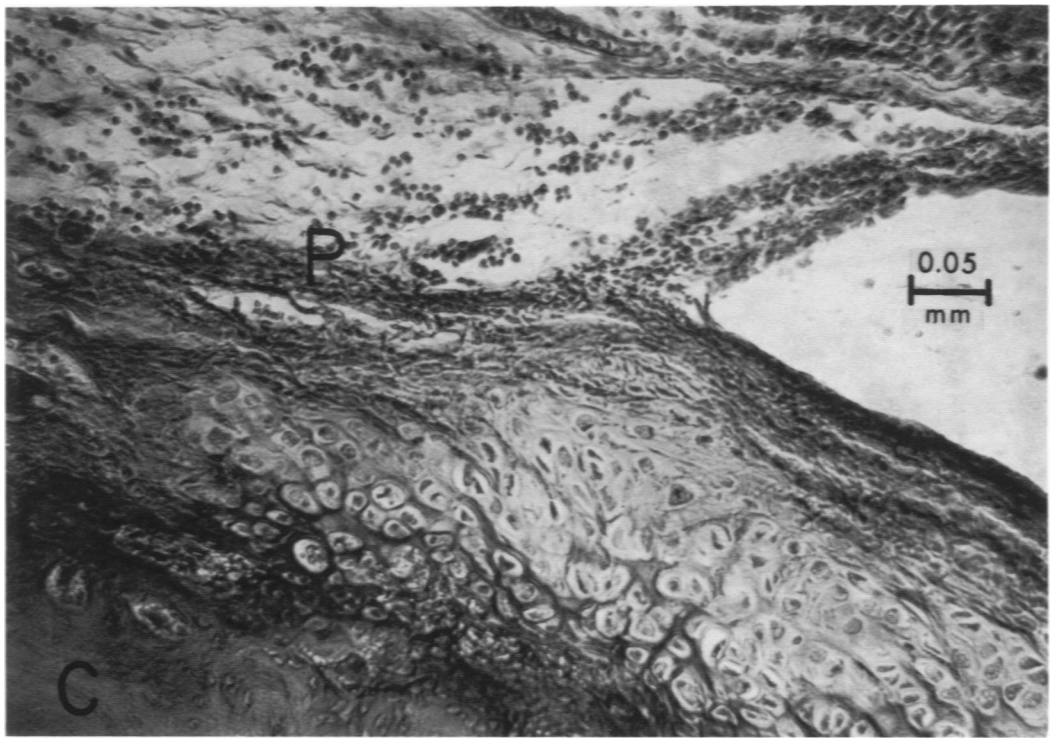
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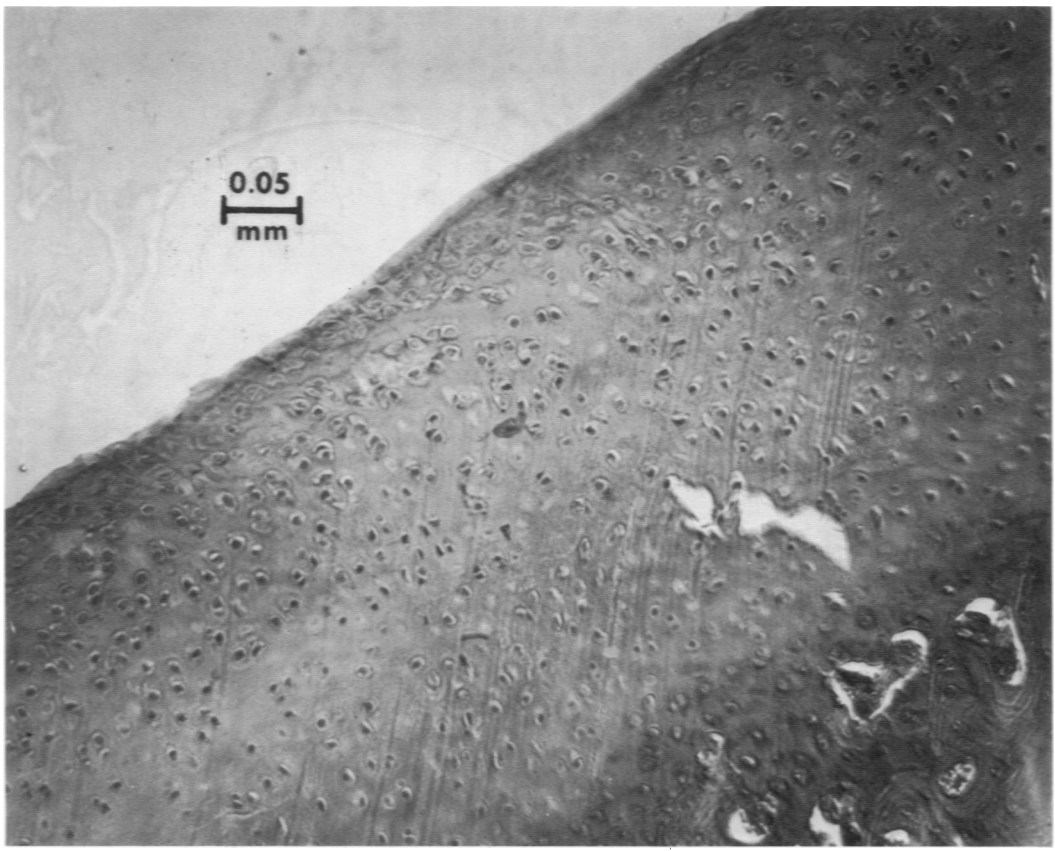

Figure 4—A histologic section of intact, normal cartilage from a saline-challenged knee. The striations are artifacts of tissue preparation. (H&E, × 140)