

## New Models of Chronic Synovitis in Rabbits Induced by Mycoplasmas: Microbiological, Histopathological, and Immunological Observations on Rabbits Injected with *Mycoplasma arthritidis* and *Mycoplasma pulmonis*

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A dose-dependent chronic synovitis was induced in rabbit knees after the intra-articular injection of both *Mycoplasma arthritidis* and *Mycoplasma pulmonis*. The inflammation progressed from an initial acute phase at 1 week, characterized by edema, infiltration of the synovium with monocytes and heterophils, and desquamation of lining cells, to a more chronic phase at 1 and 3 months, in which villus hyperplasia, lymph "nodules," mononuclear cell infiltration, fibroplasia, and collagen deposition were prominent. With one exception, mycoplasmas could no longer be cultivated from the joints 1 month postinoculation. Both mycoplasma species evoked a humoral antibody response that was more marked in synovial fluids than in peripheral blood. A cell-mediated immune reaction, as evidenced by enhanced uptake by [<sup>3</sup>H]thymidine by sensitized blood, spleen, or node lymphocytes in the presence of homologous antigen, was detected only in rabbits injected with *M. pulmonis*. Lymphocytes taken from arthritic rabbits were no more cytotoxic toward synovial cells derived from normal or arthritic rabbits than were normal lymphocytes. The models of synovitis described in this study offer a convenient probe for determining the mechanisms of mycoplasma-induced inflammation, since they require only a single injection of the initiating agent and, in addition, utilize an animal host large enough for detailed investigation into the nature of mycoplasma/synovium interactions.

Mycoplasmas are known to induce arthritic diseases in a wide variety of animal species. Swine arthritis induced by *Mycoplasma hyorhinis* (4, 24, 37) and mouse arthritis induced by both *Mycoplasma arthritidis* (17) and *Mycoplasma pulmonis* (5, 12, 28) can progress to a chronic phase that histologically resembles human rheumatoid arthritis. Although viable (13, 17, 28) and nonviable (24) antigens are known to persist in the joint tissues, the exact mechanism of synovial inflammation remains to be determined. The lack of availability of sufficient mouse synovium and the expense of working with the swine models have discouraged sequential studies to determine the nature of mycoplasma/synovial membrane interactions. Thus, the present investigation was undertaken to establish a more convenient model for the study of the pathological changes occurring within the synovial membrane as a result of infection with mycoplasmas.

Rabbits do not usually harbor mycoplasmas, although *M. pulmonis* has been isolated from animals exhibiting respiratory symptoms (21).

Previous studies in our laboratory established that a chronic mouse arthritis could be obtained after the intra-articular injection of very small numbers of *M. arthritidis* (12). This route of inoculation was therefore chosen for the rabbit in order to bypass the primary reticuloendothelial system of the host. The following report describes the histopathology of mycoplasma-induced synovitis of rabbits and preliminary microbiological and immunological characteristics of the disease.

### MATERIALS AND METHODS

**Mycoplasmas and culture procedures.** *M. arthritidis* strain 158 P10 P9 (17) and *M. pulmonis* strain JB (obtained from D. Taylor-Robinson, Medical Research Council, Clinical Research Centre, Harrow, Middlesex, England) were used throughout this study.

The mycoplasmas were grown in modified Hayflick medium consisting of Difco mycoplasma broth or agar (Difco Laboratories, Detroit, Mich.) supplemented to final concentrations of 15% (vol/vol) horse serum, 5% (vol/vol) fresh yeast extract, and 1,000 U

of penicillin G per ml (11, 30). In the case of *M. pulmonis*, the medium was additionally supplemented with 1% (vol/vol) of a sterile 2-mg/ml solution of diphosphopyridine nucleotide (DPN-102; Sigma Chemical Co., St. Louis, Mo.). Broth cultures were harvested by centrifugation, assayed for colony-forming units (CFU) as previously described (26), and stored at  $-70^{\circ}\text{C}$  before use.

**Induction of disease and collection of samples.** Female white New Zealand rabbits, 8 to 12 pounds (3.6 to 5.4 kg) in weight, were used throughout these experiments. Before injection, preimmunization sera were collected and the hind knee joints of each rabbit were carefully shaved.

The diameter of each joint was measured by averaging three caliper readings. In groups of at least three rabbits each, the right joints were injected with 0.2 ml of broth containing  $10^6$ ,  $3 \times 10^7$ , or  $10^9$  CFU of *M. arthritis* or  $10^6$ ,  $3 \times 10^7$ , or  $4 \times 10^8$  CFU of *M. pulmonis*. Six rabbits injected in the right knee with 0.2 ml of mycoplasma broth alone served as controls. The left knee of each rabbit was left uninjected.

Knee joint measurements were taken at intervals throughout 84 days. The results were expressed as percentage of increase in joint diameter as compared with the zero-time readings. Serum samples for serological studies were collected 1, 2, 4, and 12 weeks postinoculation.

One animal from each of the groups and two controls were sacrificed 1, 4, and 12 weeks postinoculation. At 4 and 12 weeks postinoculation, peripheral blood, nodes, and spleen were cultured for mycoplasmas and used for lymphocyte transformation and cytotoxicity studies. The knee joints were exposed by removing skin and pulling the patella and overlying joint capsule to one side. Joint fluid was removed for microbiological and serological studies. Pieces of tissue were dissected from the joint lining and were either transferred to 10% formalin or used for mycoplasma isolation and establishment of synovial cell lines. Fixed synovial tissues were sectioned and stained with hematoxylin and eosin by using standard procedures.

**Inflammatory response to nonviable mycoplasmas.** Rabbits were injected intra-articularly with both 50 and 250  $\mu\text{g}$  of nonviable *M. arthritis* or *M. pulmonis* antigen. The antigen used was that described for the complement-fixing (CF) antibody test. Animals were sacrificed 1 and 4 weeks postinoculation, and synovial tissues were taken for histological examination.

**Mycoplasma isolations.** Synovial fluid, when present, and synovial tissue (homogenized in 1 ml of complete mycoplasma broth) from injected and uninjected joints were transferred to blood agar or mycoplasma agar. Undiluted and  $10^{-2}$  dilutions of the suspensions were also inoculated into 5-ml amounts of mycoplasma broth and were transferred to agar after 3 days of incubation. Primary synovial cell cultures originating from explants of injected and control joints were similarly tested for the presence of mycoplasma and bacteria. All plates were examined for intervals of up to 10 days at  $37^{\circ}\text{C}$ . Mycoplasma isolates were identified by inhibition of

growth by using specific antiserum-impregnated disks.

**Serological studies.** Pre- and postinoculation serum samples and joint fluids (when available) were tested for CF antibodies according to the microtechnique of Casey (10). Antigens of *M. pulmonis* and *M. arthritis* were prepared as previously described (12, 26). All preparations were subjected to ultrasonic vibration using a Branson Sonifier (model S75, Branson Instruments Co., Stamford, Conn.) for 2-min intervals until free of viable organisms. Protein concentrations were measured according to the technique of Lowry et al. (34). Metabolic inhibiting (MI) antibodies against *M. pulmonis* were detected using the microtechnique of Taylor-Robinson et al. (41), in which inhibition of glucose metabolism was used as the indicator. The procedure of Purcell et al. (36) was used to determine the MI antibody response induced by *M. arthritis*, in which inhibition of arginine metabolism was used as the indicator.

**Lymphocyte transformations.** Rabbit blood was collected using 100 U of preservative-free heparin per ml and was separated by Ficoll-Hypaque density centrifugation according to Mansfield and Wallace (35). The resulting lymphocyte-rich preparations were washed three times in Hanks balanced salt solution containing 2% (vol/vol) fetal calf serum (FCS) by sedimentation at  $160 \times g$  for 10 min. The final cell suspensions were prepared in RPMI 1640 medium with L-glutamine (Microbiological Associates, Inc., Bethesda, Md.) containing 10% (vol/vol) FCS and were adjusted to contain  $2.5 \times 10^6$  viable cells/ml.

Spleens and lymph nodes, which were removed as soon as possible after sacrifice, were teased apart in RPMI medium and filtered through sterile 60-mesh stainless-steel gauze. The cell suspension was centrifuged at  $160 \times g$  and resuspended in 3 ml of warm 0.83%  $\text{NH}_4\text{Cl}$  [tris(hydroxymethyl)aminomethane, buffered to pH 7.2] to lyse erythrocytes (8). After centrifugation at  $160 \times g$  for 5 min, the cells were resuspended in 5 ml of fresh medium, recentrifuged, counted for viability by trypan blue exclusion (42), and finally resuspended to a concentration of  $2 \times 10^6$  cells/ml.

Lymphocyte blastogenesis was measured by two techniques. In the first procedure, detailed previously (12), 0.2-ml amounts of mycoplasma antigen containing 2 or 10  $\mu\text{g}$  of protein were added in triplicate to 2-ml amounts of the lymphocyte suspension. Tubes treated with 0.2 ml of medium served as negative controls, and tubes supplemented with 0.2 ml of a 1:50 dilution of phytohemagglutinin (PHA) (Burroughs Wellcome, Tuckahoe, N.Y.; reagent grade no. HA15) served as positive controls for T-cell blastogenesis (31). After 48 h of incubation at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  and humidity, all cultures were pulsed for an additional 24 h with 1 mCi of [ $^3\text{H}$ ]thymidine (specific activity, 6.7 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.). The harvesting procedure was as previously described (12), using a modification of the technique of Colley and DeWitt (18). The cells were washed twice in cold phosphate-buffered saline (PBS), treated with 1 N NaOH, and

given two further washings in 5% (wt/vol) trichloroacetic acid. The cells were finally dissolved in 0.5 ml of NCS tissue solubilizer and made up to 10 ml with scintillation fluid (42 ml of Liquifluor [New England Nuclear, Los Angeles, Calif.] per liter of scintillation-grade toluene). Tritium uptake, expressed as counts per minute minus background, was measured on a Nuclear-Chicago model 720 liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Counting efficiency was approximately 35%.

Lymphocyte transformation was also tested by using a microtiter modification of the above procedure. In this case, 0.2 ml of the cell suspension containing  $5 \times 10^5$  lymphocytes in RPMI medium and 10% FCS was added to each well of a flat-bottomed microtiter plate (Micro Test II tissue culture plate, Falcon Plastics, Oxnard, Calif.). Antigens also diluted in RPMI medium were added (0.025 ml per well) in triplicate, to give 0.25, 0.63, and 1.25  $\mu\text{g}$  of protein per well (i.e., approximately 1, 2.5, and 5  $\mu\text{g}/\text{ml}$ ). Positive control wells received 0.025-ml amounts of a 1:50 dilution of PHA. Negative control wells received 0.025 ml of medium. After 54 h of incubation at 37°C in CO<sub>2</sub> and humidity, the cultures were pulsed for 18 h with 0.025 ml of medium containing 0.1  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine (specific activity, 6.7 Ci/mmol). The cultures were harvested using a model A lymphocyte harvester (Otto Hiller Co., Madison, Wis.). Briefly, lymphocytes and washings from the empty wells were removed, deposited onto filter paper, and washed eight times in saline. The filter paper disks containing the washed cells were transferred to plastic scintillation counting vials (Poly Q vial; Beckman Instruments, Inc., Anaheim, Calif.) containing 10 ml of Biofluor (New England Nuclear) and mixed for 15 s on a Vortex mixer.

**Synovial cell cultures.** Monolayer cell cultures were derived from rabbit synovial tissue by an explant technique that has been previously described (27, 33, 38) and that is routinely used in these laboratories for the preparation of human synovial cell lines. All synovial cells were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air in Eagle minimum essential medium (MEM) supplemented with 10% (vol/vol) heat-inactivated FCS, glutamine (0.3 mg/ml), and penicillin (100 U/ml). The explant cultures formed confluent monolayers in approximately 3 weeks and were subsequently passaged at split ratios of 1:2.

Synovial cell lines were prepared from the synovial tissues of mycoplasma-injected and broth-injected animals at the times of autopsy. Control synovial cell lines were derived from normal, noninjected rabbits. No gross differences in morphology or growth characteristics between normal and test synovial cell lines were observed. All explant cultures were tested for the presence of cultivable mycoplasmas at confluency by isolation techniques described above.

**Cytotoxicity studies.** Assays for cytotoxic activity of rabbit lymphocytes against synovial cells were performed with an 18-h Cr<sup>51</sup>-release assay, as described elsewhere in detail for human lymphocytes and human synovial cell cultures (27). Lymphocytes

from mycoplasma- and broth-injected rabbits that were sacrificed at 1 month postinoculation were tested against second-passage normal rabbit synovial cells. Lymphocytes from rabbits that were sacrificed at 3 months postinjection were tested against both normal synovial cells and synovial cell lines derived from the appropriate mycoplasma- and broth-injected test rabbits that had been sacrificed at the 1-month time period. These cell lines were at the fifth passage after explantation at the time of assay.

## RESULTS

**Induction of Arthritis.** Both *M. arthritis* and *M. pulmonis* induced a significant joint swelling after intra-articular injection in comparison with rabbits receiving mycoplasma broth (Fig. 1 and 2). The degree of swelling was largely dose dependent and appeared to be more pronounced with *M. arthritis*. Both organisms induced a detectable reaction within 3 days. Arthritis was maximal for the first month

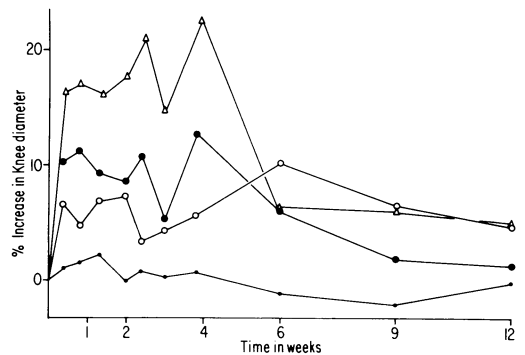


FIG. 1. Degree of joint swelling induced by the intra-articular injection of *M. arthritis* into rabbits. Animals were injected in the right knee with  $10^8$  (○),  $3 \times 10^7$  (●), or  $10^9$  (△) CFU of *M. arthritis* or mycoplasma broth (●).

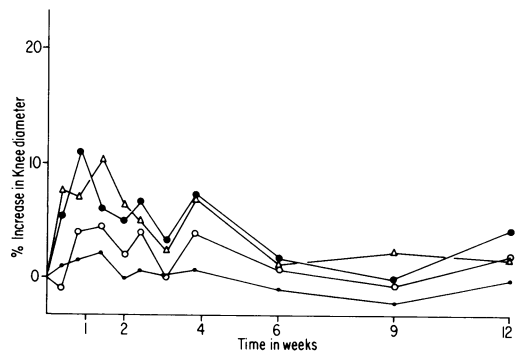


FIG. 2. Degree of joint swelling induced by the intra-articular injection of *M. pulmonis* into rabbits. Animals were injected in the right knee with  $10^8$  (○),  $3 \times 10^7$  (●), or  $4 \times 10^8$  (△) CFU of *M. pulmonis* or mycoplasma broth (●).

but persisted at lower levels for the 3-month duration of the experiments. The most severe arthritis was obtained with  $10^9$  CFU of *M. arthritidis*, in which a mean increase in knee diameter of 23% was apparent at 4 weeks post-injection. Swelling was not apparent in the uninjected knees of the animals.

Upon autopsy, both *M. arthritidis* and *M. pulmonis* appeared to induce a similar gross pathology. One week after injection, varying amounts of opaque, viscous synovial fluid were present within the injected joints. Synovial thickening with hyperemia was apparent, although the cartilage and synovial capsule appeared undamaged. One month postinoculation, the synovium was greatly thickened, and hyperemic, and often exhibited a marked villus hypertrophy (Fig. 3). Large amounts of viscous, opaque synovial fluid were present in many joints, and the joint capsule was often markedly thickened. Again, cartilage and bone appeared undamaged macroscopically. By 3 months, synovial fluid, although still opaque, was present in noticeably smaller quantities. Synovial proliferation was generally less prominent, although one animal injected with  $10^8$  CFU of *M. arthritidis* still exhibited an intense reaction. No visible changes were apparent in the uninjected joints or in those injected with mycoplasma broth.

**Histopathology.** Normal rabbit synovia were taken from the uninjected knee joints of animals that had received mycoplasma broth in the right knee joints. This tissue exhibited an inconspicuous surface layer of synovial lining cells resting on a thin fibrous base, well deline-

ated from the underlying fatty tissue (Fig. 4A and B). The contralateral joints that had been injected with mycoplasma broth exhibited at 30 days a mild diffuse inflammatory infiltration of lymphocytes and heterophilic cells. Plasma cells were not seen, and no significant proliferation of the synovial cell surface was apparent. In contrast, 7 days after the injection of rabbits with either *M. pulmonis* or *M. arthritidis*, a picture of severe active inflammation was seen. The surface layer was more cellular and its cells more epitheloid; the texture was loosened, and evidence of desquamation into the synovial space was apparent (Fig. 5A and B). The sharp delineation between synovial cells and the underlying fibrous base and stroma had disappeared. Instead, there was infiltration of a moderate to marked degree, particularly with lymphocytes and heterophilic cells that extended deeply into the congested, edematous villi.

By 30 days, the hypertrophy and cellular infiltration of the synovial villi had markedly increased (Fig. 6A). Pieces of seemingly detached necrotic villous tissue were seen in the synovial spaces. Desquamation of surface cells was still present, but it had abated somewhat. Plasma cells were prominent, but lymphocytes as well as heterophils were still present in great abundance. In addition, discrete subsurface lymphoid "nodules" had appeared. The capillaries and venules that contained masses of inflammatory cells were even more conspicuous than at 7 days. The endothelial lining cells were prominent and epitheloid, and inflammatory cells were seen to penetrate the vessel walls (Fig. 6B).

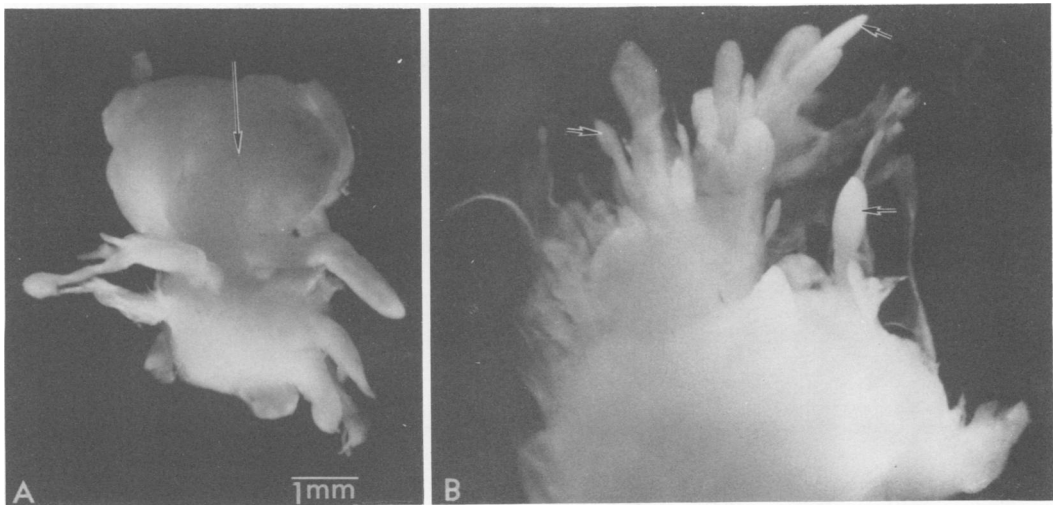


FIG. 3. Synovial villi produced in knee joints 1 month postinjection with  $10^6$  CFU of *M. pulmonis* (A) or  $4 \times 10^8$  CFU of *M. pulmonis* (B).

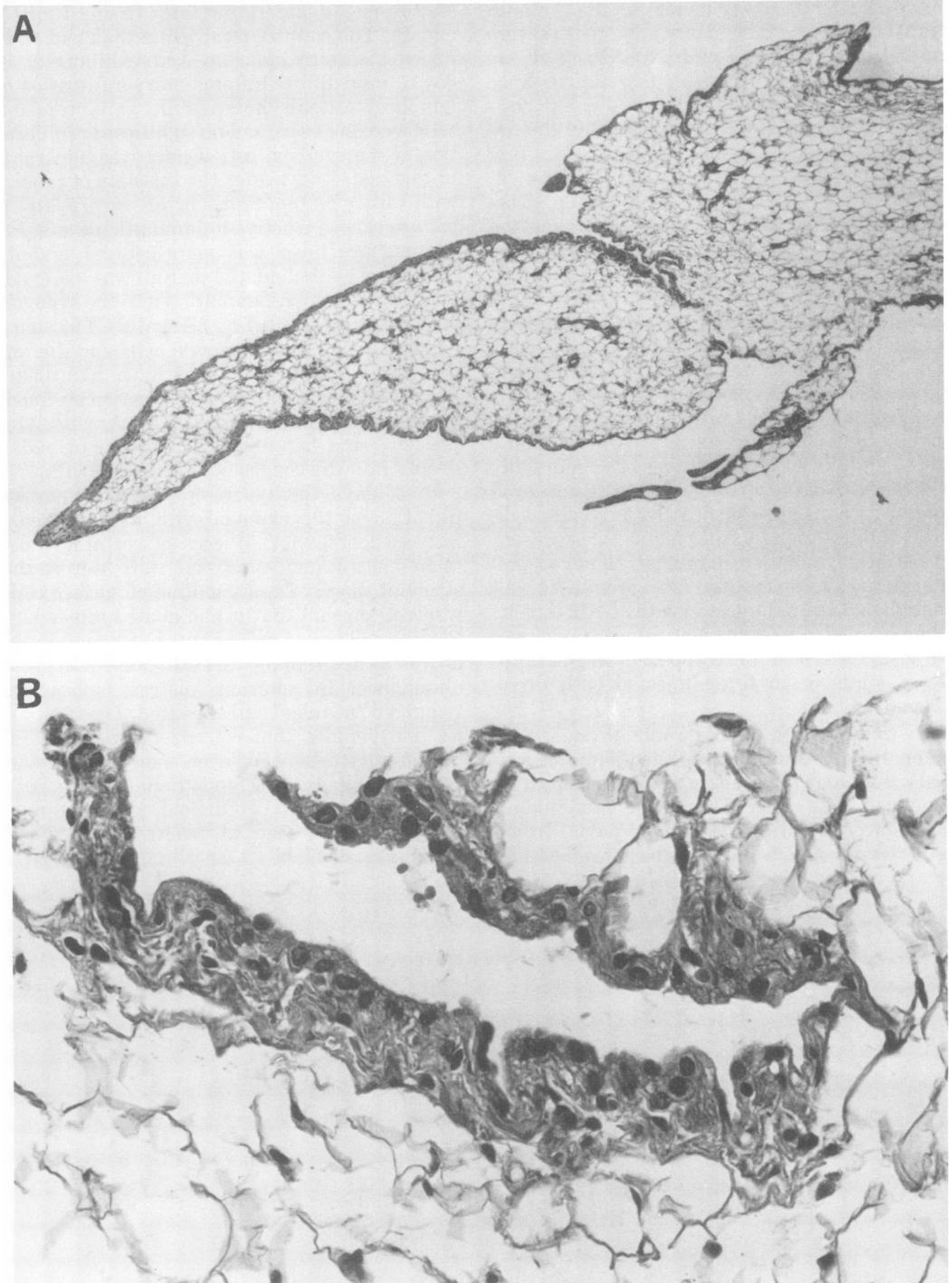


FIG. 4. (A) Normal rabbit synovium ( $\times 45$ ). Note simple outline, straight surfaces, uniform, fairly well-vascularized acellular stroma, and thin, even surface layer. (B) Normal rabbit synovium ( $\times 450$ ). Note surface and subsurface synovial lining cells; the delicate, even fibrous base is well delineated from the underlying stroma.

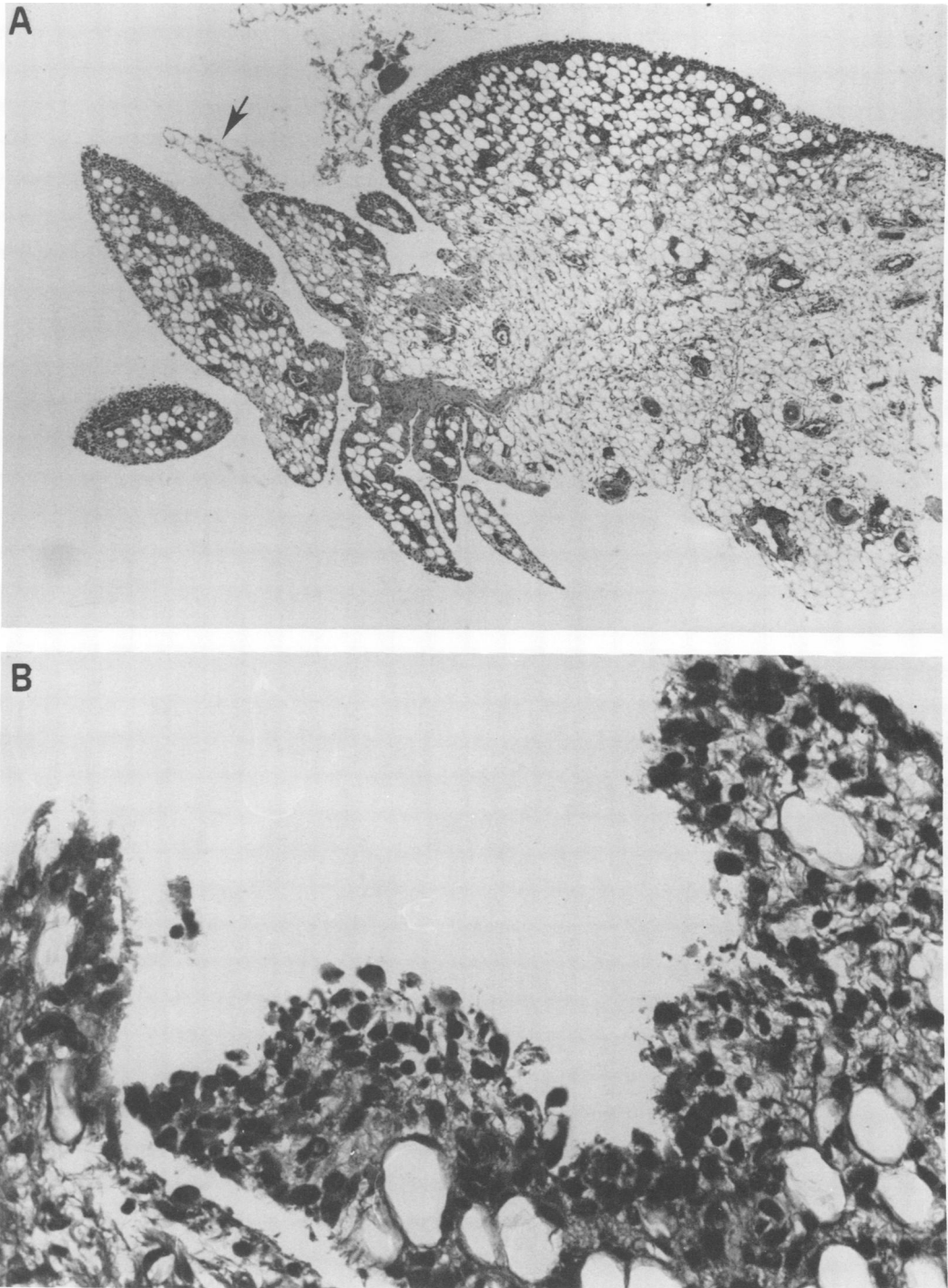
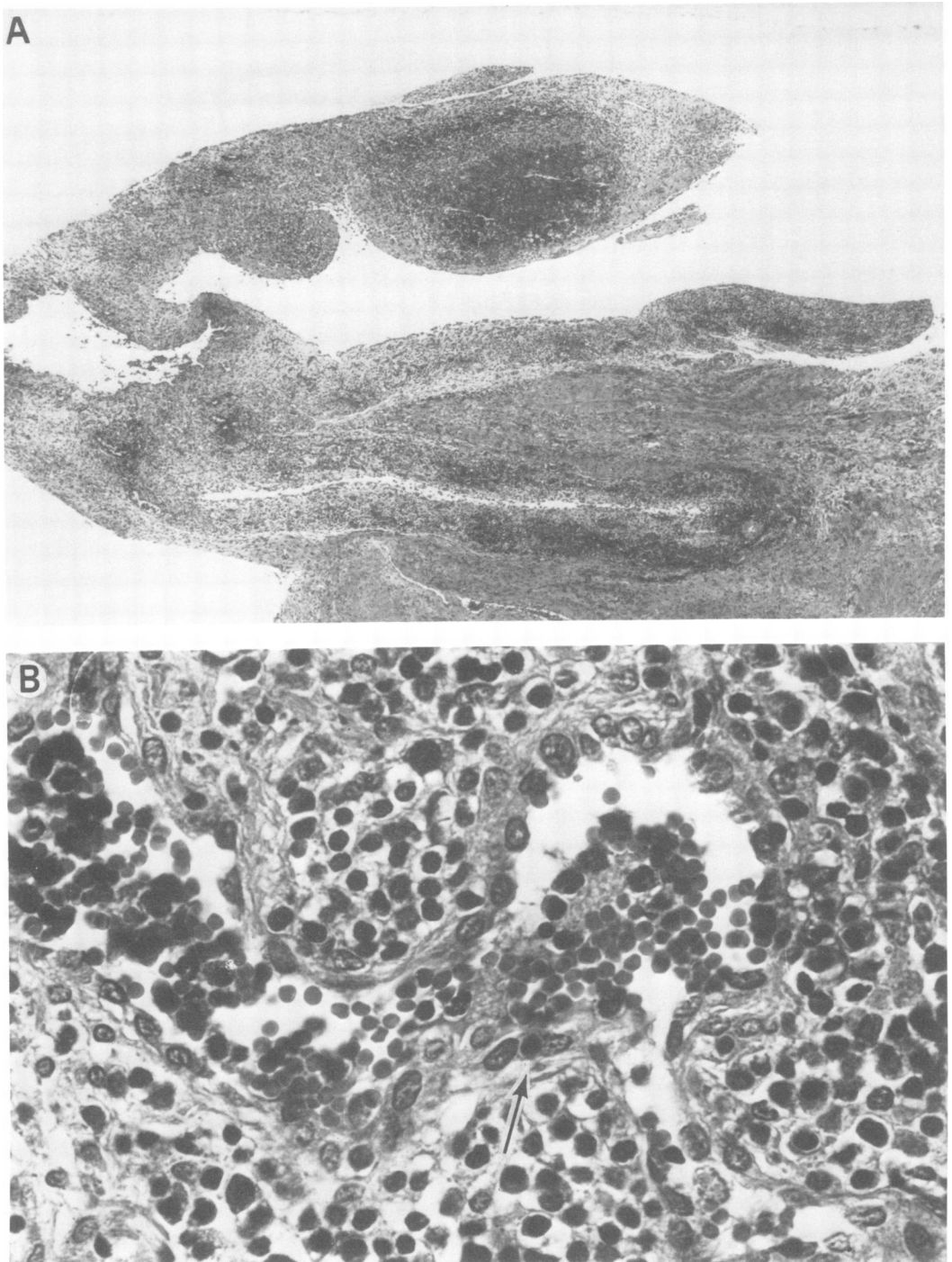


FIG. 5. (A) Rabbit synovium, 7 days after intra-articular injection of  $10^8$  CFU of *M. arthritis* ( $\times 45$ ). Note proliferation of villi, hypertrophy of surface layer, irregular delineation from underlying tissue, increased vascularity and cellular infiltrate, and desquamated necrotic villous fragments (arrow). (B) Rabbit synovium, 7 days after intra-articular injection with  $3 \times 10^7$  CFU of *M. pulmonis* ( $\times 450$ ). Note increased cellularity of surface layer, loosening of texture, and cellular desquamation; edema and lymphocytic infiltration is seen beneath the surface.



**FIG. 6.** (A) Rabbit synovium, 30 days after injection of  $10^9$  CFU of *M. arthritis* ( $\times 45$ ). Increased villous hypertrophy, massive cellular infiltration, and formation of lymphoid "nodules" are apparent. (B) Rabbit synovium 30 days after intra-articular injection of  $10^6$  CFU of *M. arthritis* ( $\times 720$ ). Massive infiltration of stroma by lymphocytes, plasma cells, and heterophilic leukocytes is seen. Vascularity is strikingly increased, and the lumen contains masses of inflammatory cells. The vascular endothelium is distinctly hyperplastic; epitheloid, and inflammatory cells are seen to penetrate endothelial lining (arrow).

By 90 days, certain differences between rabbits injected with *M. pulmonis* and those injected with *M. arthritidis* had become apparent. Inflammatory changes persisted in both groups, and macrophages were present in large numbers. However, in the *M. pulmonis*-injected rabbits, inflammation was less and fibrotic foci were present (Fig. 7A and B). In the *M. arthritidis* rabbits, the inflammation persisted unabated, and lymph nodules were still seen near the surface (Fig. 8A and B).

Of particular interest was the finding that the injection of nonviable organisms induced a lesser but qualitatively similar inflammatory response in rabbit synovium. Although the reaction was most marked at 7 days, it was still apparent by 2 months. The prominent feature of this response, particularly in the *M. pulmonis*-injected rabbits, was the occurrence of foci of marked synovial cell proliferation and nests of plasma cells and lymphocytes.

The inflammatory changes produced by viable organisms were not entirely limited to the injected joints. Thus, after 7 days the synovial surface layer of the uninjected contralateral knee was more cellular than normal, and a mild to moderate infiltration by heterophil and mononuclear cells was observed, particularly in the case of *M. pulmonis*. These changes had largely abated by 30 and 90 days, except for rare foci of lymphocytes, macrophages, and fibrosis.

**Isolation of microorganisms.** Bacteria were not recovered at any time from the tissues of injected or uninjected rabbits. Control rabbits and uninjected joints were free of cultivable mycoplasmas at all time periods. Similarly, mycoplasmas were never recovered from spleen, node, or peripheral blood lymphocyte suspensions used for the cell-mediated immunological studies.

Using direct transfer to agar, *M. arthritidis* was recovered from the synovial fluid of one joint injected 1 week previously with  $10^6$  CFU of *M. arthritidis*. Preculture in mycoplasma broth resulted in the isolation of mycoplasmas from the homogenized synovial tissues of all injected rabbits and from the synovial fluids of two of the four rabbits tested (one rabbit received  $10^6$  CFU of *M. arthritidis*, and the other received  $3 \times 10^7$  CFU of *M. pulmonis*). One month postinoculation, *M. arthritidis* was recovered from only one joint fluid by preculture in broth. All other cultures were negative, indicating a very low level of persistence of organisms. All attempts to recover mycoplasmas from rabbits 3 months postinoculation failed.

Explants of synovium taken from the joints of arthritic and control rabbits 1 and 3 months

postinjection were incubated in medium containing penicillin as the only antibiotic. The resulting synovial cells were cultured for mycoplasmas when confluency was reached. Mycoplasmas were not isolated from any of these cell lines.

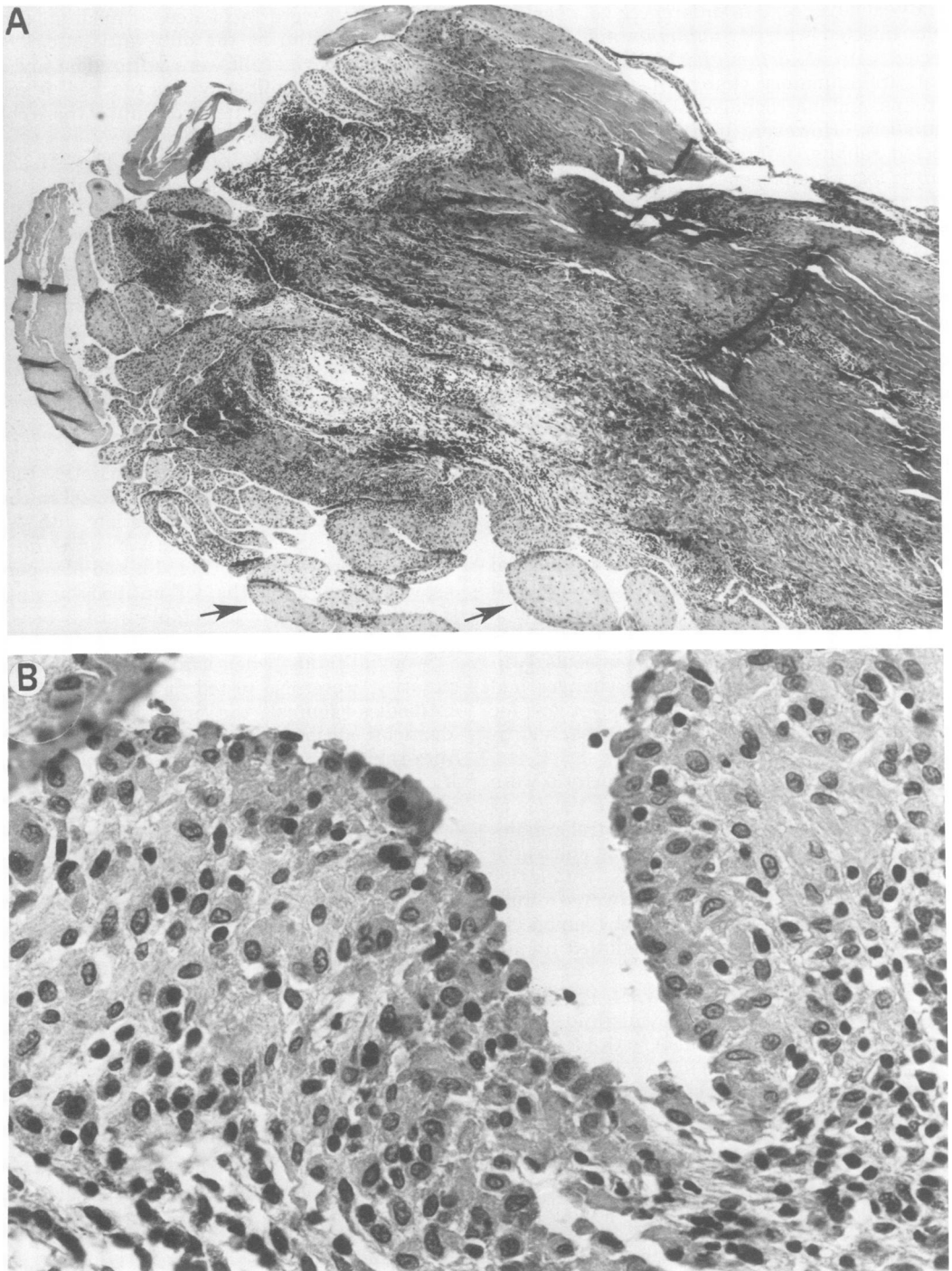
**Humoral antibody responses.** The MI and CF antibody levels in the serum of rabbits injected with *M. arthritidis* and *M. pulmonis* are summarized in Fig. 9 and 10, respectively. Preinjection sera and sera from control rabbits injected with mycoplasma broth did not exhibit antibody levels against either *M. arthritidis* or *M. pulmonis*. Since there was no correlation between individual antibody levels and dose or organisms injected, mean values of all rabbits at each time period were recorded.

MI antibodies against *M. arthritidis* were first detected in the sera of rabbits 2 weeks postinoculation and persisted for 3 months (Fig. 9). In contrast, MI antibodies developed in the sera of mice 1 week after injection with *M. pulmonis*. CF antibodies against *M. arthritidis* were present at 1 week (Fig. 10) and also persisted through 3 months. CF antibody against *M. pulmonis* was not detectable until 2 weeks postinjection.

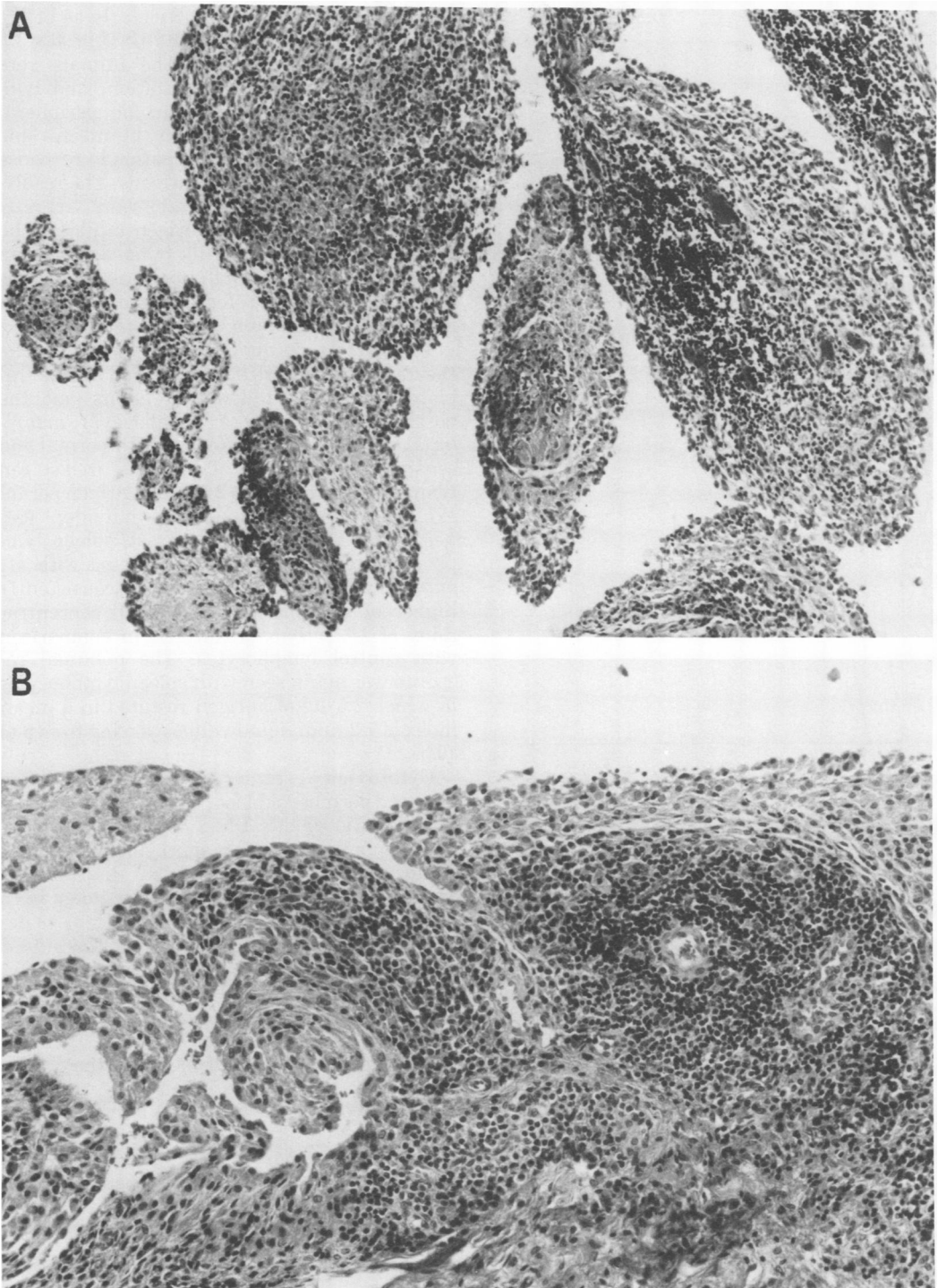
Synovial fluids, when available in sufficient quantity, were also tested for the presence of antibodies against the injected organisms. Mean MI antibody levels of five fluids taken from rabbits injected 1 month previously with *M. arthritidis* were  $>1:192$  (range,  $<10$  to  $>1,280$ ). The corresponding mean CF antibody level was  $>461$  (range, 64 to  $>1,280$ ). Synovial fluids taken from rabbits injected 1 month previously with *M. pulmonis* exhibited a mean MI titer of  $>710$  (range, 320 to  $>1,024$ ) and a mean CF titer of 880 (range,  $<10$  to 2560). Thus, higher levels of antibody were usually present in joint fluids than in the peripheral circulation.

**Cell-mediated immune responses.** Spleen and node lymphocytes collected from rabbits 1 month postinoculation with mycoplasma broth, *M. arthritidis*, or *M. pulmonis* were examined by the macrotest for transformation after incubation with PHA or mycoplasma antigens (Table 1). The lymphocytes of all rabbits responded to PHA, individual indexes ranging from 6.7 to 73.8 for spleen lymphocytes and 6.9 to 245.7 for node lymphocytes. The uptake of [ $^3$ H]-thymidine by spleen and node lymphocytes from control rabbits was suppressed in the presence of both concentrations of *M. arthritidis* antigen (individual indexes ranged from 0.1 to 0.4) but was somewhat enhanced in the presence of *M. pulmonis* antigen (indexes ranged from 1.0 to 4.2). The mitogenic potential of *M.*





**FIG. 7.** (A) Rabbit synovium 90 days after injection of  $4 \times 10^8$  CFU of *M. pulmonis* ( $\times 45$ ). Persistent villous hypertrophy with focal fibrotic changes in several involved villi (arrow). Although abundant residual active inflammatory cell infiltration is seen, some villi are beginning to exhibit decreased cellularity (arrows). (B) As for (A) ( $\times 450$ ). The surface layer has remained markedly thickened and not well delineated from underlying stroma. Most of the cells accounting for increased cellularity of the surface layer are macrophages, plasma cells, and hypertrophied fibroblasts, lymphocytes having become less conspicuous than at 30 days. Desquamation of surface cells has decreased somewhat.



**FIG. 8.** (A) Rabbit synovium 90 days after injection of  $10^6$  CFU of *M. arthritis* ( $\times 110$ ). Hypertrophied villi, massive inflammatory cellular infiltration, and persistence of lymphoid "nodules" are apparent. (B) As for (A) ( $\times 180$ ). Note venule in center of lymphoid nodules and persistent hyperplasia of vascular endothelium at its right. The thickened synovial surface layer contains macrophages, plasma cells, and fibroblasts.

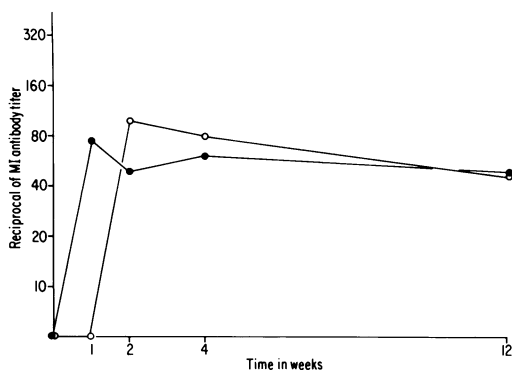


FIG. 9. Serum MI antibody response of rabbits to the intra-articular injection of *M. arthritidis* (O) or *M. pulmonis* (●).

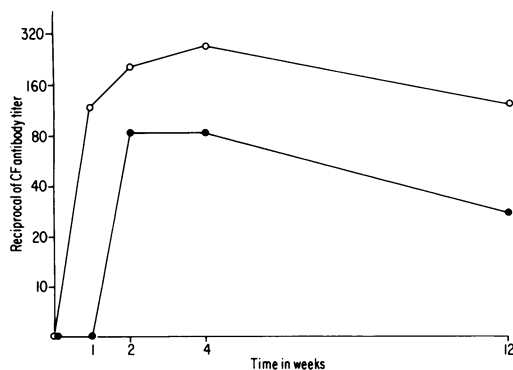


FIG. 10. Serum CF antibody response of rabbits to the intra-articular injection of *M. arthritidis* (O) or *M. pulmonis* (●).

*pulmonis* antigen for normal rat and mouse lymphocytes has already been described (12, 25).

Lymphocytes taken from rabbits injected 1 month previously with *M. arthritidis* did not transform in the presence of *M. arthritidis* antigen. In fact, a suppression of lymphocyte activity was apparent. Thus, spleen lymphocytes exposed to 5  $\mu\text{g}$  of antigen per ml exhibited a mean index of 0.2 (individual values ranged from 0.03 to 0.6). In contrast, lymphocytes from rabbits injected with *M. pulmonis* exhibited a marked blastogenic response to *M. pulmonis* antigen in comparison to the response obtained with control lymphocytes. Thus, spleen lymphocytes exposed to 5  $\mu\text{g}$  of antigen per ml exhibited a mean index of 16.8, with individual values of 7.2 to 29.4. This response was therefore comparable with that obtained with PHA.

To develop a microtechnique for measurement of blastogenesis, a test group of rabbits was set up in which *M. pulmonis* and *M. ar-*

*thritidis* were given intra-articularly as before in doses of  $3 \times 10^7$  and  $4 \times 10^8$  CFU or  $3 \times 10^7$  and  $10^9$  CFU, respectively. The animals were sacrificed 1 month postinoculation, and lymphocytes from nodes, spleen, or peripheral blood were examined by both the macro- and microtechniques for transformation in response to PHA and mycoplasmal antigens. The results obtained by the two procedures were virtually identical. *M. arthritidis* antigen inhibited the uptake of [ $^3\text{H}$ ]thymidine by lymphocytes from all rabbits, and *M. pulmonis* antigen was markedly mitogenic for lymphocytes taken from sensitized rabbits.

The microtechnique was utilized to examine the responses of spleen, node, and peripheral blood lymphocytes taken from rabbits 3 months postinoculation. As observed above, *M. arthritidis* antigen was inhibitory to both normal and sensitized lymphocytes. Thus, sensitized spleen lymphocytes exposed to 2.5  $\mu\text{g}$  of antigen per ml exhibited a mean index of 0.4 (individual values from 0.09 to 1.1). In contrast, spleen lymphocytes taken from rabbits injected with *M. pulmonis* continued to exhibit a consistently higher blastogenic response to all concentrations of *M. pulmonis* antigen in comparison with control lymphocytes. The maximal response was again seen with spleen lymphocytes in which 2.5  $\mu\text{g}$  of antigen resulted in a mean index of 7.2 (individual values varying from 6 to 10.3).

**Cytotoxicity studies.** Lymphocytes taken from the peripheral blood, spleens, and nodes of the arthritic rabbits 1 and 3 months postinjection demonstrated no greater cytotoxic activity against normal rabbit synovial cells than did analogous lymphocyte preparations taken from broth-injected control animals. Spleen, node, and blood lymphocytes from animals sacrificed at 3 months were also tested for cytotoxic activity against synovial cells derived from the arthritic joints of the *M. arthritidis*- and *M. pulmonis*-injected rabbits that had been sacrificed at 1 month. Synovial cells from the mycoplasma broth-injected animals (also prepared at the 1-month time interval) served as controls. No increased cytotoxicity activity against synovial cells from the arthritic animals by lymphocytes from the mycoplasma-injected rabbits compared with lymphocytes from control rabbits was detected.

## DISCUSSION

The present study has shown that both *M. arthritidis* and *M. pulmonis* induce an intense inflammatory reaction in the synovial membrane of rabbits after intra-articular injection.

TABLE 1. Blastogenic responses of rabbit lymphocytes 1 month postinoculation with *M. arthritis* or *M. pulmonis*

Source of lymphocytes	Mean cpm and blastogenic indexes obtained after exposure to:					
	$^3\text{H}$ thymidine control	PHA	<i>M. arthritis</i> antigen		<i>M. pulmonis</i> antigen	
			1 $\mu\text{g}^a$	5 $\mu\text{g}$	1 $\mu\text{g}^a$	5 $\mu\text{g}$
Control rabbits (2) <sup>b</sup>						
Spleen	2,451	61,484 (46.5) <sup>c</sup>	992 (0.4)	891 (0.3)	9,455 (2.6)	9,183 (2.9)
Nodes	869	60,511 (69.6)	109 (0.1)	117 (0.1)	1,439 (1.7)	2,067 (2.4)
<i>M. arthritis</i> -infected rabbits (3)						
Spleen	6,181	92,204 (27.2)	454 (0.2)	522 (0.2)	NT <sup>d</sup>	NT
Nodes	2,685	125,049 (140.4)	256 (0.3)	440 (0.4)	NT	NT
<i>M. pulmonis</i> -infected rabbits (3)						
Spleen	4,275	70,154 (18.2)	NT	NT	53,888 (13.3)	67,313 (16.8)
Nodes	2,228	35,219 (13.8)	NT	NT	8,571 (3.8)	14,008 (5.2)

<sup>a</sup> Amount of antigen per milliliter of lymphocyte culture.

<sup>b</sup> Number of rabbits.

<sup>c</sup> Mean blastogenic index calculated by averaging the individual indexes from each rabbit.

<sup>d</sup> NT, Not tested.

Histopathologically the lesion progressed from an initial acute phase at 1 week to a more chronic phase at 1 month, which was characterized by massive villus hyperplasia of the synovial membrane with lymphocyte, macrophage, and plasma cell infiltration and fibroplasia. Although some healing was evident by 3 months, active inflammation persisted, especially in animals injected with *M. arthritis*. Preliminary observations (Cole et al., unpublished data) indicate that the arthritic response can persist considerably longer than 3 months. Thus, one of four rabbits injected 14 months previously with  $3 \times 10^7$  CFU *M. arthritis* still exhibited a severe macroscopic arthritis. The explanation for the development of a mild transient inflammation in the uninjected contralateral knee joints of infected rabbits is not apparent at this time, since viable mycoplasma were not recovered from these tissues.

Detailed comparisons between the arthritis induced by *M. arthritis* and *M. pulmonis* were not possible in this study due to the limited number of animals examined. However, the synovial reaction observed with *M. pulmonis* appeared to be more proliferative in nature, with noticeably less edema and infiltration with inflammatory cells in comparison

with the lesions induced by *M. arthritis*.

Of particular interest was the finding that with one exception the synovial tissues and fluids were free of cultivable organisms by 1 month despite the marked inflammation. The relatively high levels of MI antibody in the serum and synovial fluids of injected rabbits could explain the elimination of viable organisms. In contrast, the survival of mycoplasmas within the joints of Swiss mice many months after administration (13, 17) correlates with the minimal MI antibody response of these mice to the organisms (15, 16). It remains to be established whether viable mycoplasmas or persisting nonviable antigen are necessary for the chronicity of the murine models of this disease.

Previous studies in our laboratories (12-14) have shown that both *M. arthritis* and *M. pulmonis* induce a cell-mediated reaction in infected mice as evidenced by enhanced uptake of  $^3\text{H}$ thymidine by sensitized lymphocytes exposed to homologous antigen. This reaction may contribute to the chronic inflammatory response due to persistence of the sensitizing antigens. In the rabbit models, a significant cell-mediated immune response to mycoplasma antigen was detected in animals injected with *M. pulmonis* but not in animals injected with

*M. arthritis*. These results may be related to the reported in vivo and in vitro immunosuppressive properties of *M. arthritis* (6, 7, 20, 32). Whether a cell-mediated immune reaction plays a role in the inflammatory response is not yet known and must await further studies to define persistence of mycoplasma antigen in the rabbit articular area. Recent studies have indicated that the inhibitory effects of *M. arthritis* on [<sup>3</sup>H]thymidine uptake by lymphocytes in vitro can be eliminated by heat treatment of the antigen. The use of this antigen resulted in the detection of a cell-mediated immune response in a test rabbit sensitized against *M. arthritis* (Cole et al., unpublished observations).

Studies by other investigators utilizing a variety of antigen-induced arthritides of rabbits have shown that inflammation is dependent upon an immune reaction to persisting antigen (19, 23). Additional evidence for the participation of a cell-mediated immune response in antigen-induced arthritis was the finding that joint fluids of arthritic rabbits contained macrophage migration-inhibitory factor (39) and that lymphokines produced in vitro exhibited an inflammatory reaction after intra-articular injection (1). The low level of stimulation of normal rabbit lymphocytes by nonviable *M. pulmonis* antigen is consistent with previous studies which indicated a mitogenic potential for this species toward mouse lymphocytes (12, 25). The observation that nonviable *M. pulmonis* but not *M. arthritis* induced villus hypertrophy after intra-articular injection may be related to the mitogenic potential of the former organism. Thus, mitogen-mediated lymphokines or other toxic mycoplasma constituents may play a role in the initiation of the inflammatory response. The rabbit joint clearly responds to toxic products as evidenced by the synovitis produced as a result of repeated injections of streptolysin-S or filipin, agents that cause liberation of lysosomal enzymes (45, 46).

The cytotoxicity studies reported here were designed primarily to detect occult infection of joint tissue with noncultivable mycoplasmas. Specific lysis of virus-infected target cells by immune lymphocytes has been a useful technique in investigations of cell-mediated immunity to viral agents in mice (22) and man (40). The lack of cytotoxic activity of lymphocytes from arthritic animals against synovial cells derived from mycoplasma-injected joints supports the conclusions of the isolation data that viable mycoplasmas do not remain in the joint space. These data do not preclude, however, the possibility that mycoplasma antigen is retained in the joint tissues, since non-proliferating ma-

terial would be diluted out during the passage of the cell lines.

A secondary aspect of the cytotoxicity studies was to provide preliminary data about the possible role of autoimmune phenomena in mycoplasma-induced rabbit synovitis. An autoimmune reaction could be initiated by a mycoplasma-induced alteration of host tissues or by the occurrence of antigens common to parasite and host (9, 29). Evidence of an immune reaction to host synovial antigens in *M. pulmonis*-induced arthritis of mice has been reported by other investigators (29). Our very brief investigation of this question in the rabbit models was entirely negative. However, these results may reflect either the limited number of time points studied after initiation of the disease, the loss of tissue specific antigens by the synovial target cells during maintenance in tissue culture, or failure to use a suitable sensitized lymphocyte population, i.e., synovium-derived lymphocytes. It is not known whether the mild inflammatory reaction in contralateral uninjected knees represents evidence of an autoimmune reaction. Thus, the role played by autoimmunity in mycoplasma-induced rabbit synovitis remains to be determined and will be the subject of further investigation in this laboratory.

The ability of mycoplasmas to produce chronic arthritis in rabbits is of interest in view of the observations of Webb et al. (43), who showed that an intense inflammation was produced when pools of herpes simplex virus contaminated with *Acholeplasma laidlawii* were injected intra-articularly into rabbits. Later studies by these investigators using pure pools of herpesvirus resulted in a much less intense arthritis, thus suggesting a synergistic effect due to the mycoplasma (3).

The arthritis model described in this report represents an important new probe for defining the mechanisms of both acute and chronic inflammation. Although the model is artificial in respect to route of injection, thus limiting studies related to virulence factors, it nevertheless utilizes agents that are known arthritogenic organisms in other animal species. Unlike antigen-induced arthritis (23) or arthritis induced by streptolysin-S or filipin (44, 45), in which either a presensitized host or repeated injections are required, the mycoplasmal disease requires only a single injection of the agent. Furthermore, viable organisms disappear rapidly despite continued inflammation. The chronic arthritis induced in rabbits by a single intravenous injection of either viable (2) or nonviable (46) *Erysipelothrix* appears to be comparable with the mycoplasmal model. All of these organisms exhibit a tropism for joint tissues in

their natural hosts.

The availability of both normal and arthritic synovial tissue will permit detailed work to be undertaken on the mechanisms of synovial inflammation. Furthermore, our observations that two biologically distinct mycoplasma species, i.e., *M. arthritidis* and *M. pulmonis*, induce an intense inflammatory response in rabbits provide an opportunity for defining possible diverse inflammatory pathways.

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