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# *Cytochemical Demonstration of Constitutive $H_2O_2$ Production by Macrophages in Synovial Tissue From Rats With Adjuvant Arthritis*

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**Generation of toxic oxygen metabolites by inflammatory cells is considered to be one of the mechanisms by which inflammation produces tissue injury. This concept is based on *in vitro* studies of purified leukocyte populations because it has not been possible to assess production of these metabolites in tissues. In order to determine whether or not inflammatory cells in tissue generate $H_2O_2$, the authors modified an earlier cytochemical method for the localization of $H_2O_2$.[1] The incubation medium consists of 0.5 mM $CeCl_3$ in a Hepes-buffered balanced salt solution with $Cl^-$ as the only anion. Synovial tissue from the knees of normal and 16-day adjuvant arthritic rats was incubated in this medium for 30 minutes and then fixed and processed for electron microscopy. No $H_2O_2$ reaction product was visible in normal synovium. In contrast, patchy deposits of $H_2O_2$ reaction product were seen adjacent to a subpopulation of synovial lining macrophages in synovium from inflamed knee joints. These data show that rat synovial macrophages are capable of generating $H_2O_2$ when appropriately stimulated and that such a stimulus is present in adjuvant arthritis. (Am J Pathol 1988, 130:120–125)**

THE TISSUE INJURY that results from chronic inflammation has been attributed, at least in part, to the formation and release of toxic oxygen metabolites.[2,3] Inflammatory cells *in vitro* increase their oxygen consumption when appropriately stimulated and convert much of this oxygen to superoxide anion and $H_2O_2$ (reviewed by Babior[4] and Badwey et al[5]). It has been hypothesized that similar events occur *in vivo* and that generation of highly reactive oxygen metabolites is an important means by which stimulated macrophages and neutrophils mediate host defenses and inadvertent tissue injury.[5] Additionally, for macrophages, the ability to generate reactive oxygen species upon stimulation is considered to be an indicator of activation.[6,7]

The data upon which these hypotheses are based have been obtained in vitro from purified populations. It is not clear how relevant these phenomena are to events *in vivo* because measuring systems lack specificity and free radical scavengers prevent direct measurement of reactive oxygen in tissue. In contrast, cytochemical methods can detect localized sites of enzyme activity even when average or specific activity is low. $H_2O_2$ production can therefore be theoretically detected by cytochemical means at the level of individual cells within tissue.

Briggs et al previously described a method[1] for the ultrastructural localization of sites of $H_2O_2$ generation by neutrophils. This technique takes advantage of the fact that cerium cations ($Ce^{3+}$) react with $H_2O_2$ to form cerium perhydroxide, an electron-opaque precipitate. We modified the previously published technique by incorporating cerium into a balanced salt solution in which excised tissue may be maintained at 37 C for an hour or more without morphologic alteration. We thus can dectect by cerium precipitation any $H_2O_2$ generated within tissues derived from normal or pathologic states. Using this method on synovial tissue from animals with adjuvant arthritis, a model of chronic inflammation, we found that $H_2O_2$ generated by phagocytic cells could indeed be detected by cytochemical means in an inflammatory

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lesion. Macrophages in structurally intact synovial lining tissue from rats with adjuvant arthritis generated $H_2O_2$ spontaneously, whereas macrophages in corresponding areas from control rats did not. Furthermore, in contrast to adjacent neutrophils, infiltrating macrophages in disrupted regions of inflamed synovial tissue did not spontaneously produce $H_2O_2$.

## Materials and Methods

Adjuvant arthritis was induced in inbred male Lewis rats (Charles River, Kingston, NY) by injection of complete Freund's adjuvant into the left foot pad. The animals were sacrificed by lethal injection of Nembutal 16 days after induction of the disease and tissue for study taken from the contralateral knee. Knee joints from normal and arthritic rats were opened by incising the quadriceps muscle transversely and exposing the synovium from the patella to the tibia. The whole infrapatellar fat pad, including the patella, was dissected free of the infrapatellar ligament and quickly placed in ice-cold buffer containing 140 mM NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM KCl, 15 mM Hepes, 0.1% glucose, pH 7.3. While in buffer, most of the patella was removed, leaving only a small piece attached for orientation. The subsynovial fat was also cut away, and the sides were trimmed to form a trapezoid, the short side containing the patella fragment. Dissection of tissue in this buffer was necessary because preliminary experiments indicated that cerium ions rapidly and irreversibly precipitated with most anions other than chloride. Furthermore, we found that macrophages were more sensitive than neutrophils to the toxic effects of the reaction medium described in the original paper.[1] Macrophages usually adhere to the culture dish; but when incubated with the original reaction mixture, they rounded up and lifted off the plastic surface. At 0.5 mM $CeCl_3$, in Hepes buffer, adherent macrophages remain attached and well spread for at least 2 hours, and synovial tissue so incubated retained normal morphology.

The tissue was washed in several changes of fresh buffer, allowed to come to room temperature, and placed in incubation medium, which consisted of the above buffer, to which had been added 10.0 mM 3-amino-1,2,4-triazole (ATZ), 0.5 mM $CeCl_3$, and 0.71 mM NADH. The tissue was incubated in this medium for 30 minutes at 37 C, after which time it was washed again in buffer, fixed in 2% glutaraldehyde, postfixed in 1% osmium, and then processed by standard techniques for electron microscopy. Care was taken to embed the tissue with the patella fragment at the front of the block so that sections were taken for examination from corresponding areas of each block and were always at right angles to the synovial membrane.

In some control experiments, a postfixation acid wash (pH 6.0) was used as described by Briggs et al[1] for removal of cerium hydroxide that might have formed during incubation at alkaline pH. No change in reaction product distribution was seen. In other control experiments, glutaraldehyde was added to the reaction mixture so that we could see whether damaged cells released materials such as phosphate that could mimic $H_2O_2$ reaction product. No cerium precipitate was seen in tissue that was exposed to glutaraldehyde after the initial wash and dissection in the medium described above. Cerium precipitate was also not formed when catalase was included in the reaction mixture and ATZ (an inhibitor of catalase) omitted.

### Distribution of $H_2O_2$ Reaction Product in Synovial Lining Tissue of Control Rats

The normal control synovial membrane was two to three cells thick and constituted a boundary zone between collagenous tissue and the joint space (Figure 1). The cells were loosely spaced in this boundary zone and appeared not to make junctions with one another in the manner of an epithelial membrane. The macrophagelike cells were generally found at or near the membrane surface, and fibroblasts were a little deeper. Projections from both cell types mingled at the interface of the membrane with the joint space.

Synovia from control animals did not stain with cerium unless the tissue had been traumatized during dissection. If most of the fat pad and patella was not removed from the tissue, a cloudy precipitate formed during the cerium incubation. This precipitate adhered to the upper and lower surfaces of the tissue but had a different appearance in the electron microscope than the $H_2O_2$ reaction product found in tissue from animals with adjuvant arthritis. When present, $H_2O_2$ reaction product formed a heavy, amorphous deposit, whereas the artifactual precipitate had a microcrystaline structure. Although the source of the precipitate is uncertain, we hypothesize that cerium reacted with released glycosaminoglycans, because it can precipitate with chondroitin sulfate *in vitro*.

### Distribution of Reaction Product in Inflamed Synovia

The morphology of the synovial lining adjacent to the patella varied from sample to sample. In some samples the membrane was similar to control synovia, with a discrete limiting membrane containing macrophagelike and fibroblastlike cells separating the

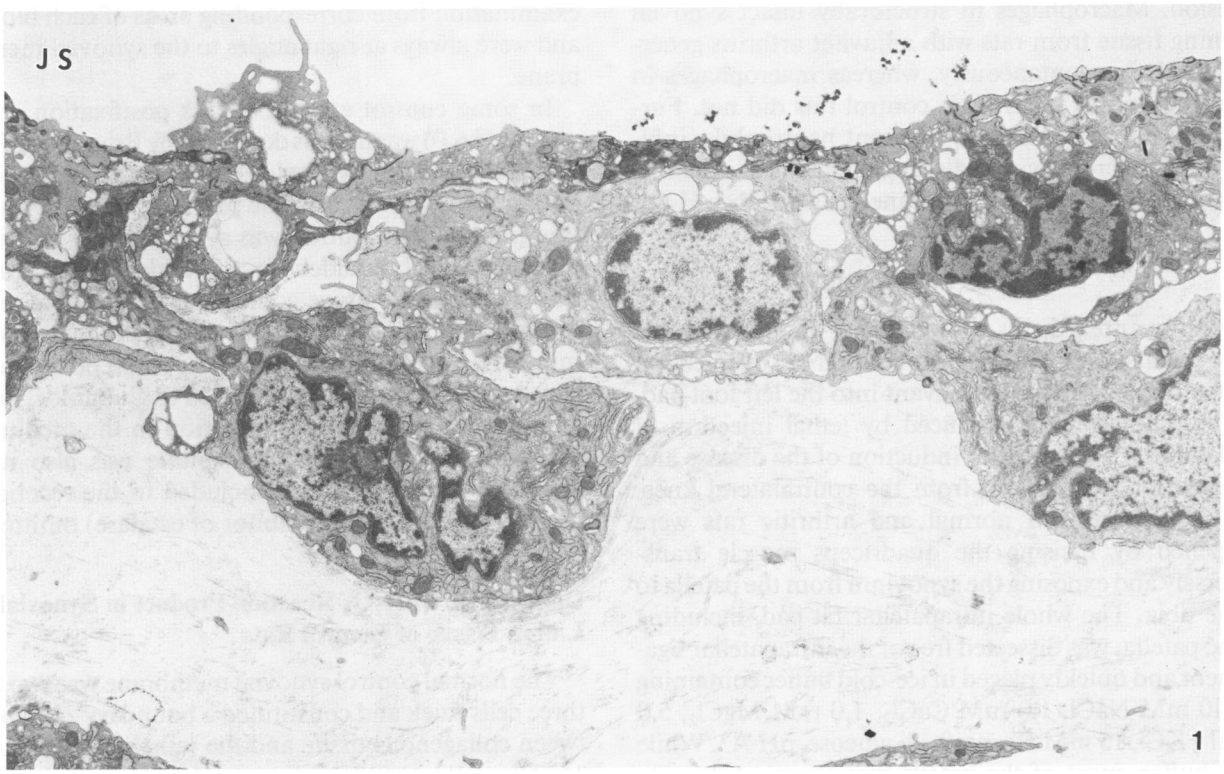


**Figure 1**—The typical appearance of normal synovial lining tissue. The cells are irregularly shaped, with long processes, and are a mixture of macrophages and fibroblasts on a loose collagenous matrix. This tissue had been incubated in reaction medium, but reaction product is not seen. A small amount of nonspecific precipitate (*arrowhead*) is visible in the joint space (*JS*). (×5200)

collagenous tissue from the joint space. The only morphologic differences between these specimens and tissue samples from control rats were 1) the presence in inflamed synovium of numerous ruffles projecting from macrophages into the lumen of the joint space, suggestive of cell activation and 2) a wider spacing between cells, suggestive of edema (Figure 2). In these samples dense masses of electron-opaque $H_2O_2$ reaction product were found around macrophages (Figures 2 and 3), with smaller amounts diffusely distributed in the adjacent collagenous matrix. A similar relationship between reaction product and fibroblast-like cells was not observed.

In other specimens from rats with adjuvant arthritis, the surface architecture in the patellar region was totally disrupted by fibrin deposits (Figure 4). Infiltrating neutrophils and macrophages were visible within and on top of the fibrin masses. $H_2O_2$ reaction product was visible adjacent to neutrophils in these disrupted regions, suggesting that inflammatory stimuli were still present, even though infiltrating inflammatory macrophages did not respond with an oxidative burst.

The ability to generate $H_2O_2$ was not limited exclusively to phagocytes from animals with adjuvant arthritis. One knee pad, dissected from an apparently normal control rat, contained a blood clot, as if the animals had bruised its knee sometime before sacrifice. Copious amounts of $H_2O_2$ reaction product were observed not only between lining macrophages as was seen in the arthritic rats but also around some of the macrophages in deeper tissue regions. Additional reaction product was also seen around blood vessels, particularly in association with pericytes (Figure 5). This phenomenon was also seen from time to time in other specimens although never in glutaraldehyde-prefixed controls, suggesting that the precipitate was a result of an enzymatic process.

## Discussion

The results reported here illustrate the utility of the cerium-based cytochemical procedure for localizing sites of $H_2O_2$ production in tissue where $H_2O_2$ has been suspected of functioning as an inflammatory mediator.[8] The possibility exists that its use can be extended to other organs such as kidney and lung where reactive oxygen metabolites are thought to contribute to cell injury.[9,10] A major hurdle to be overcome in the adaptation is the propensity of $Ce^{3+}$ to precipitate with many anions. Thus, for avoidance of artifactual

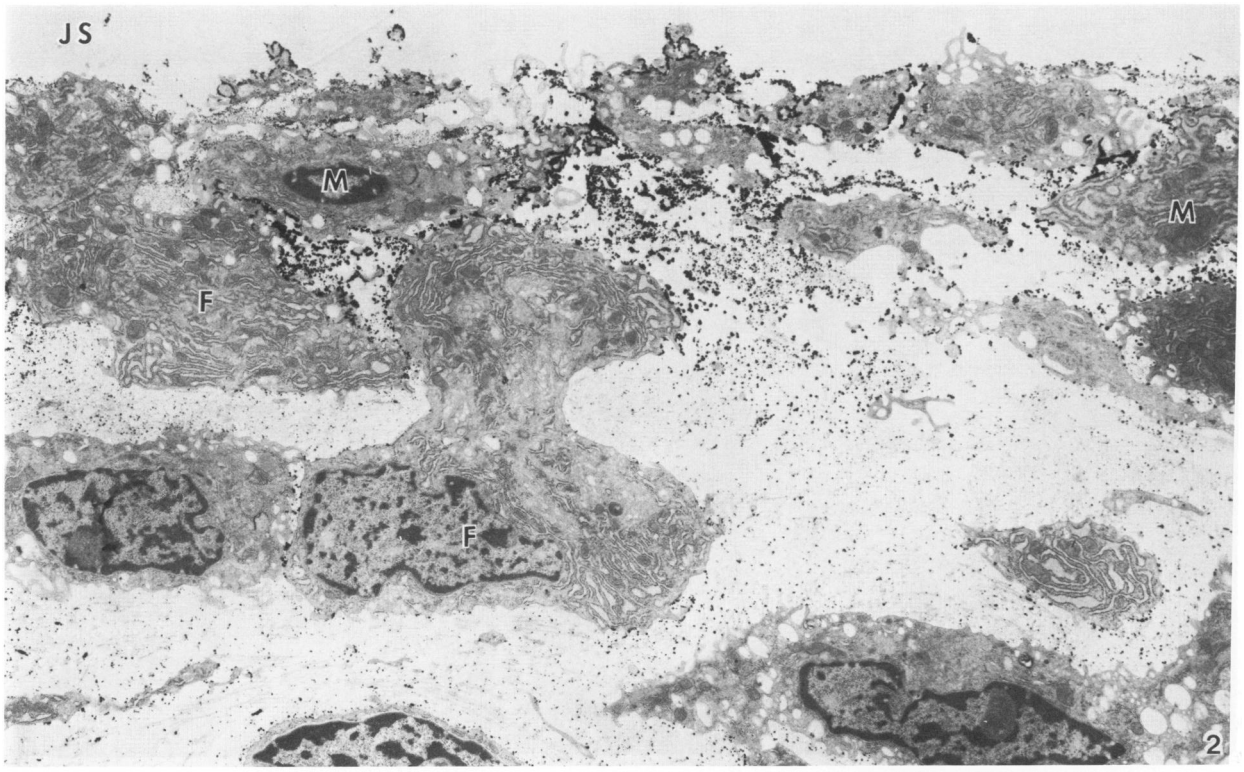


**Figure 2**—A corresponding area taken from an animal with adjuvant arthritis shows a profusion of electron-opaque reaction product around and between superficially disposed macrophages (*M*) with little or none around fibroblasts (*F*). (×5200)

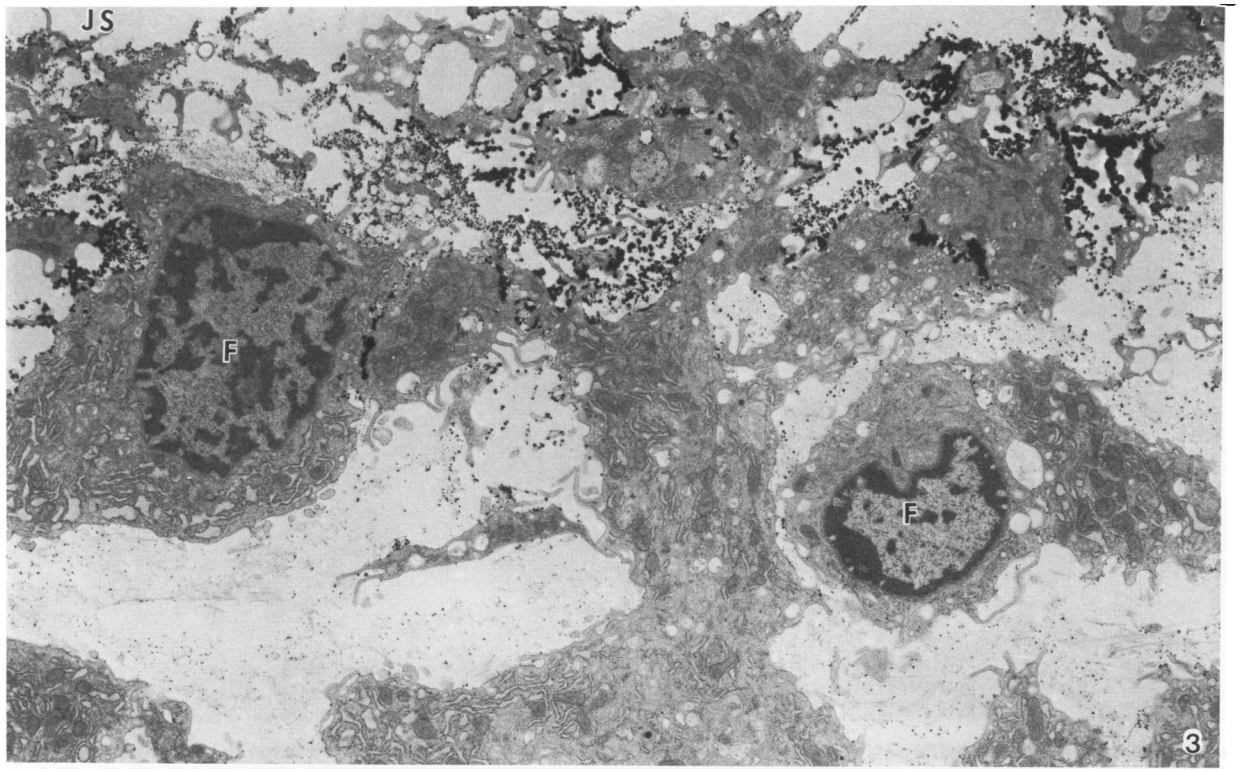


**Figure 3**—A similar view at a slightly higher magnification of synovial lining cells from another animal with arthritis. The pattern of reaction product deposition is the same as that shown in Figure 3. (×6700)

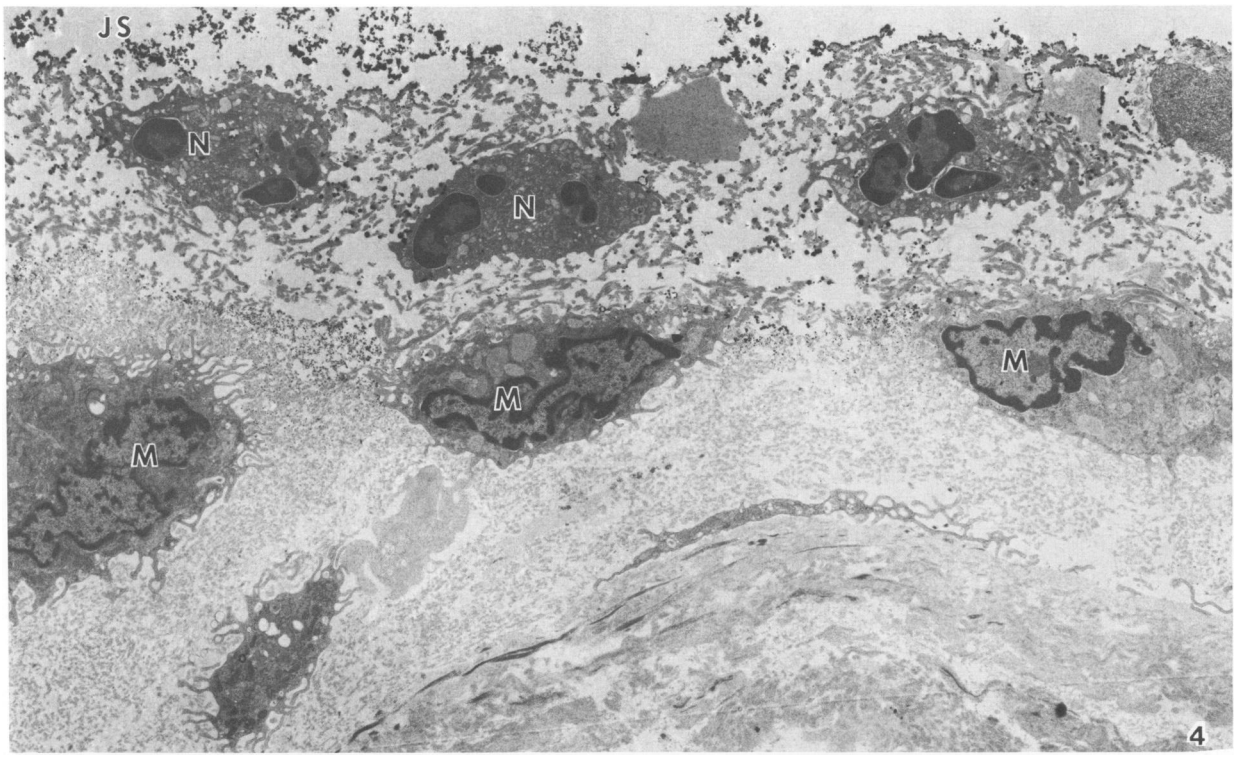


**Figure 4**—the same region of synovium from another animal with adjuvant arthritis in which there has been extensive disruption of the normal architecture, deposition of fibrin, and infiltration by macrophages (*M*) and neutrophils (*N*). $H_2O_2$ reaction product is visible in the zone occupied by the latter but not the former. (×4200)

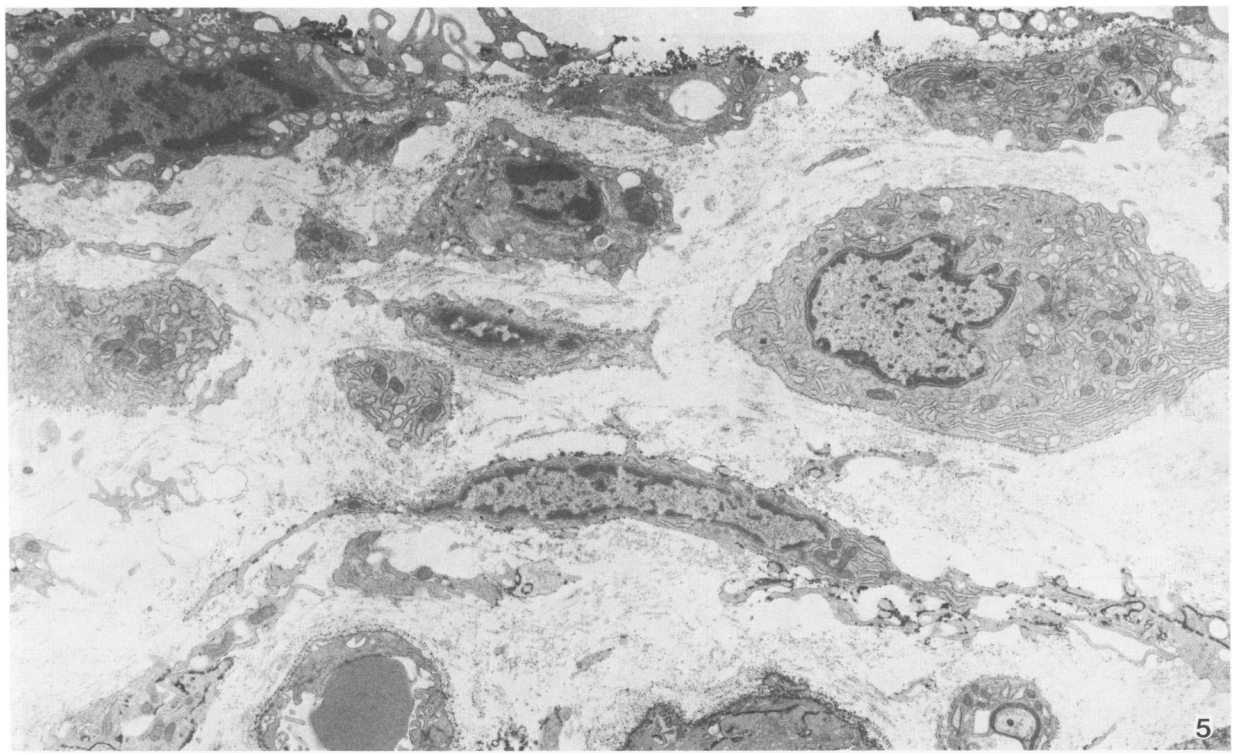


**Figure 5**—The synovium was obtained from a control animal in which a blood clot was found in the joint space upon dissection. Reaction product was visible in the synovial lining layer like that found in the arthritic animals. Additional reaction product was also visible in and around macrophages that were embedded more deeply in the tissue. These macrophages were never spontaneously reactive in tissue from arthritic animals or tissue from control animals that had been mishandled during dissection. (×5800)

precipitates, the tissue or organ of interest must be rinsed or perfused before contact with the reaction mixture. The other issue is the likelihood of inflammatory cells being stimulated by the amount of handling required to process the tissue. Methods and techniques for handling would have to be tailored for each application of the cerium procedure.

Using this method on excised tissue from a model of chronic inflammatory disease we have shown that $H_2O_2$ generated by phagocytic cells can be detected by cytochemical means. Macrophages in structurally intact synovial lining tissue from rats with adjuvant arthritis were shown to generate $H_2O_2$ constitutively, whereas macrophages in corresponding areas from control rats did not. The latter cells were, however, capable of generating large amounts of $H_2O_2$ when appropriately stimulated or when the tissue was slightly crushed or stretched, so that some cells were damaged during preparation. In contrast, infiltrating macrophages in structurally disturbed regions of the synovial tissue did not spontaneously produce $H_2O_2$ even though adjacent neutrophils did. Interestingly, these cells did not respond to mechanical damage in the same way as resident cells in control synovia, suggesting that their ability to respond to stimuli of $H_2O_2$ generation was in some way impaired.

The ability of macrophages to generate reactive oxygen metabolites when appropriately stimulated is reported to be related to their state of activation.[6] In mice, the species in which activation has been most thoroughly studied, resident macrophages produce relatively little superoxide anion, compared with elicited macrophages such as those found infiltrating an inflammatory lesion. In contrast, activation in rats results in a decrement in stimulated superoxide anion release by macrophages derived from peritoneum, spleen,[12] and lung (unpublished data). This difference between the species might be related to the respective abilities of these cells to produce prostaglandins, molecules which depress superoxide production through activation of adenylate cyclase. In mice prostaglandin production decreases with activation,[13] whereas in rats it increases.[14]

Thus, the differences that we saw between lining cells and infiltrating cells might reflect real differences in the distribution of endogenous stimuli to which the cells were responding. Alternatively, those differences might represent different degrees of responsiveness to endogenous stimuli by resident and infiltrating macrophages. Future work in which the relationship between $H_2O_2$ reaction product formation and the presence of endogenous stimuli such as immune complexes is determined will help elucidate this question.

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