Altered Acid Hydrolase Activities in Rheumatoid Synovial Cells in Culture

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EARLIER CYTOCHEMICAL STUDIES of normal and rheumatoid synovial membranes showed markedly increased acid phosphatase and oxidase activities in the lining cells of the rheumatoid membrane.[1] Acid phosphatase activity, evident in cytoplasmic granules, serves as a marker for lysosomes—subcellular organelles first described by deDuve and shown to contain numerous hydrolases active at an acid pH.[2,3] Homogenates of rheumatoid synovial membranes contain a much higher content than normal of acid phosphatase, and two other lysosomal hydrolases —cathepsin[4] and β-glucosaminidase.[5]

Synovial cells derived from normal and rheumatoid membranes have been grown in tissue culture by a number of workers.[6–8] Phase-contrast microscopy and hematoxylin and eosin stains have failed to reveal consistent differences between the normal and rheumatoid synovial cells in culture. Bartfeld[6] reported multinucleated giant cells, bizarre nuclei, a wide variation in cell size, and coarsely granular cytoplasm of cells in late rheumatoid cultures. It is not certain whether these changes are unique to rheumatoid cells, for they were observed in normal cells in culture by Castor and Dorstewitz.[7] In an extensive study, Stanfield and Stephens[8] could detect no significant morphologic differences.

It would be important to demonstrate differences between rheumatoid and normal cells in culture. We have investigated the possibility that rheumatoid synovial cells in culture maintain the increased enzymatic activities that they show in tissue sections. Cytochemical methods were used, and cells were incubated for activities of three lysosomal acid hydrolases—acid phosphatase, β-glucosaminidase, and aryl sulfatase.

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Materials and Methods

Synovial membrane samples were obtained at arthrotomy from 5 patients with rheumatoid arthritis, and from 4 patients with normal synovia who were undergoing operation for repair of injury to the meniscus. All samples were transported to the laboratory in chilled nutrient medium (Dulbecco-Vogt modification of Eagle's medium plus 10% calf serum) and were explanted within 4 hr. The cultures were grown in sterile disposable plastic Petri dishes at 37° C. in an atmosphere of 10% CO_2. Subcultures were made by using a 0.25% trypsin solution to remove the cells to fresh Petri dishes, with or without coverslips, as needed. For each experiment, normal and rheumatoid cells were handled in an identical manner with respect to subculture inoculum, days in subculture, and medium changes. The cells were studied following growth periods of 5–18 days after subculture—as early as the second, and as late as the seventh subculture.

Cytochemistry

Cells grown on glass coverslips or on plastic Petri dishes were washed 3 times in normal saline and fixed for 3 min. in cold 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4.[9] After 3 rinses in distilled water, incubation for enzyme activities was carried out at 37° C. in the following media:

Acid phosphatase was visualized by using Gomori's lead medium [10] with β-glycerophosphate as substrate for 25 and 45 min., or in Barka and Anderson's [11] azo-dye medium with naphthol AS-TR phosphate as substrate for 45 and 60 min. The freshly prepared lead medium was heated at 37° C. for 1 hr. and filterd twice immediately prior to use.

Glucosaminidase was demonstrated by utilizing Hayashi's [12] N-acetyl-β-glucosamine naphthol AS-LC medium by incubation for 60 and 90 min.

Aryl sulfatase activity was visualized by using a p-nitrocatechol sulfate medium at pH 5.5 for 60 and 90 min.[13]

Control preparations were incubated in substrate-free media.

Results

Both rheumatoid and normal cultures contained multinucleated giant cells, bizarre nuclei, and variations in cell size; however, these changes were more prevalent in the rheumatoid cultures, as reported by Bartfeld.[6] In unfixed cells examined under the microscope, the coarse granularity of the rheumatoid cells was striking. The rheumatoid cell plates always contained more cell debris and amorphous material in the supernatant medium than did the normal cell cultures.

The pattern of acid hydrolase activity differed in rheumatoid and normal cells. In addition to lysosomal staining, diffuse cytoplasmic staining was a common occurrence in rheumatoid cells (Fig. 1). Nuclear staining, an artifact attributed to diffusion of reaction product, was not prominent in these specimens. Even after prolonged incubation, diffuse staining was not evident in normal cells, although large numbers of lysosomes were stained (Fig. 2). The incubation time required to visualize lysosomes in the rheumatoid cultures was shorter than in the normal (Fig. 3 and 4). In the normal cell cultures the lysosomes measured ap-

proximately 0.5 μ; much larger lysosomes, greater than 1.5 μ, were often seen in the rheumatoid cell cultures (Fig. 5 and 6). Similar staining patterns were observed with cell cultures incubated for acid phosphatase (by both the lead and azo-dye procedures), aryl sulfatase, and glucosaminidase activities. The latter preparations showed the lowest level of activity.

To test the phagocytic capacity of the cultured cells, we added the heme protein horseradish peroxidase (Sigma Chemical Co.) to the culture medium (3 mg./ml. and 5 mg./ml.), and at intervals of 1, 2, and 3 hr. after addition of the protein, the cells were fixed and stained by 2 different benzidine procedures [14,15] to visualize the peroxidase. No uptake of peroxidase was demonstrable in either the normal or the rheumatoid cells.

Discussion

These observations reveal distinct differences between rheumatoid and normal synovial cells grown through as many as 7 subcultures, for a total of 4 months. Increased acid hydrolase activities have consistently been observed in cells derived from 5 different rheumatoid membranes which were compared with cells from 4 normal membranes. Previous light- and electron-microscopic studies demonstrated marked increases in lysosomal and oxidative enzyme activities in rheumatoid synovial membranes.[16] Studies of rheumatoid synovial slices showed enhanced oxidative metabolism and glycolysis compared to normal membrane slices,[17] and homogenates of rheumatoid synovia revealed high levels of some glycolytic enzymes.[18] The present study indicates that rheumatoid synovial cells in culture retain the enhanced lysosomal enzyme activity that is so striking a feature of the whole tissue. It is not known why this altered and enhanced metabolism persists through 4 months of culture and subculture. The possibility that an infectious agent is transferred during repeated passages in culture must be considered. It also remains to be determined whether this is a specific characteristic of rheumatoid arthritis cells or whether cells from other types of inflamed synovia also show these changes.

Whether synovial cells in tissue culture are derived predominantly from the lining cells or the subsynovial fibroblasts has not been resolved. Vaubel,[19] who was the first to grow synovial cells in culture, considered them to be different from fibroblasts and called them synovioblasts. Castor, Prince, and Dorstewitz, who have extensive experience in this field, considered cells cultured from synovia, skin, and periosteal and serosal surfaces to be "monotonously alike" morphologically and pre-

ferred to designate them all "fibroblasts."[20] Later, Castor and Muirden[21] reported that under the electron microscope, cultured synovial cells "resembled a mixture of Type A and B cells" which is the nomenclature used[22] to describe fine structural features of two types of lining cells in the human synovial membrane. We cannot say with certainty whether the cells in tissue culture are derived from the lining cells or basal fibroblasts; however, the cells in culture continue to secrete hyaluronate, which appears to arise in the synovial membrane largely from the lining cells.[23]

An important finding in the present study was the diffuse cytoplasmic staining in the rheumatoid cells. This observation is difficult to evaluate because diffuse cytoplasmic staining may represent either artifactual leakage of enzymes (or reaction product) from highly active lysosomes, or the in-situ visualization of enzymes that have become solubilized in the living cell. In our material, the absence of nuclear staining—the most common type of artifactual staining due to diffusion—argues against the possibility of artifact but does not rule it out.

The provocative suggestion has been made that in the living cell "fragile" lysosomes "leak" acid hydrolases and other material into the cytoplasm, and that the release of these components from the cell provokes tissue damage and an inflammatory response in rheumatoid arthritis[24] and in other disorders.[25,26] As yet there is no evidence that this actually occurs in the rheumatoid joint. Elevated levels of lysosomal hydrolase activity in rheumatoid synovial fluids[27–29] have been offered as evidence for such a release.[24] However, high levels of hydrolases may be released from degenerating inflammatory cells or sloughed lining cells in the synovial fluid, rather than from cells in the rheumatoid synovial membrane. Indeed, rheumatoid synovial fluids also show high levels of glycolytic and Krebs-cycle enzymes,[30,31] suggesting that if leakage is occurring, it is not confined to lysosomes.

Weiss and Dingle[32] described generalized cytoplasmic staining for acid phosphatase in fibroblasts grown in culture following the addition of "antilysosomal" globulin to the medium. This was interpreted as the visualization of enzymes released from the lysosomes into the cytoplasm. These cells died within minutes after addition of the antilysosome globulin. Allison, Harington, and Birbeck[25] reported diffuse cytoplasmic staining in macrophages after the uptake of silica. These cells died in about 30 hr. Uptake of nontoxic materials did not induce this phenomenon. In our experiments the rheumatoid cells were not dying cells, and replicate plates could be further subcultured.

The underlying intracellular events leading to this altered metabolic

activity are also unknown. It has been suggested that enhanced phagocytosis (endocytosis) of products from the joint fluid by the rheumatoid lining cells, with incomplete intracellular digestion, results in the formation of increased numbers of lysosomes.[16] In tissue culture, continued phagocytosis of joint components is excluded. Our peroxidase experiments indicate that the phagocytic capacity of both the cultured normal and rheumatoid cells is limited. At the present time we may conclude that histochemical procedures can distinguish between rheumatoid and normal cells grown in tissue culture, but interpretation of the diffuse staining is uncertain.

Summary

We investigated the possibility that rheumatoid synovial cells grown in tissue culture maintain the increased lysosomal enzyme activity that they show in sections taken from the whole tissue. Cells from 4 normal and 5 rheumatoid synovial membranes were grown on coverslips in nutrient medium with calf serum added. After brief aldehyde fixation, cells were incubated to determine acid phosphatase, aryl sulfatase, and glucosaminidase activities. Rheumatoid cells were readily distinguished from the normal cells by their enhanced and altered staining for these lysosomal hydrolases. This was seen as early as the second subculture and as late as the seventh—after 4 months of continuous subculture. Why this altered metabolic activity persists through repeated passages in tissue culture is not known.

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[*Illustrations follow*]

Legends for Figures

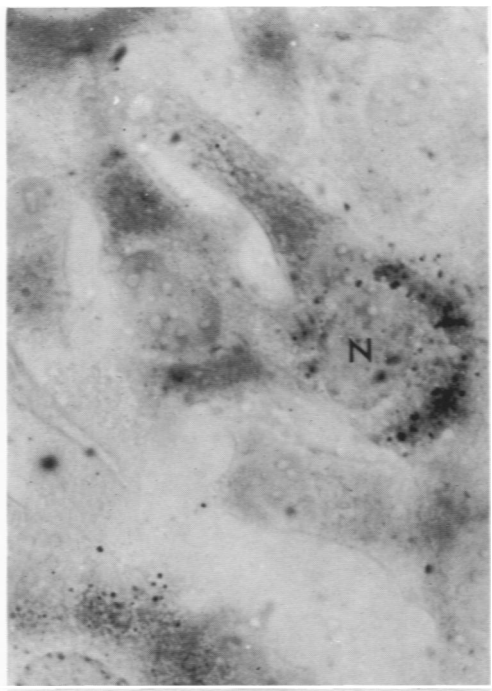
All figures are of synovial cell cultures fixed in 2.5% glutaraldehyde for 3 min.

Fig. 1. Rheumatoid synovial cells. Incubated for acid phosphatase activity for 40 min. Diffuse cytoplasmic staining is prominent. Nucleus (N). × 700.

Fig. 2. Normal synovial cell. Incubated for aryl sulfatase activity for 90 min. With prolonged incubations large numbers of lysosomes are seen in normal cultures; they are found around the nucleus and in the long cellular processes. Nucleus (N). × 1000.

Fig. 3. Rheumatoid synovial cell incubated for acid phosphatase activity at the same time and under the same conditions as preparation shown in Fig. 4. Many lysosomes are seen in this brief incubation. They vary in size from approximately 0.5 to 1 μ. Nucleus (N). × 1000.

Fig. 4. Normal synovial cells incubated for acid phosphatase activity in a Gomori medium for 25 min. Only a few small lysosomes are seen (arrows). Nucleus (N). × 1000.



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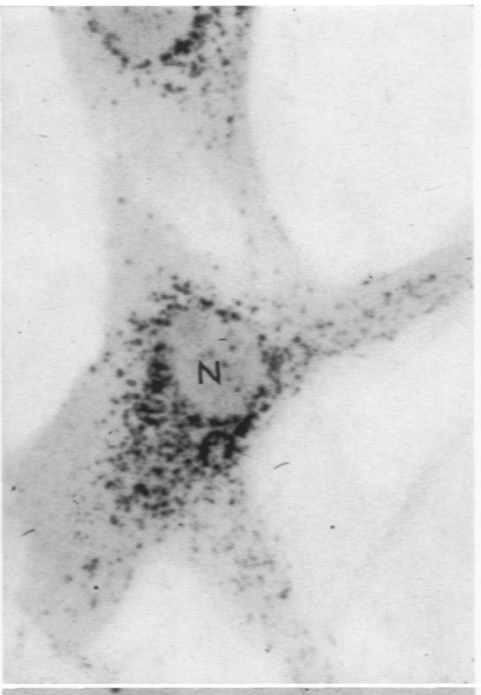

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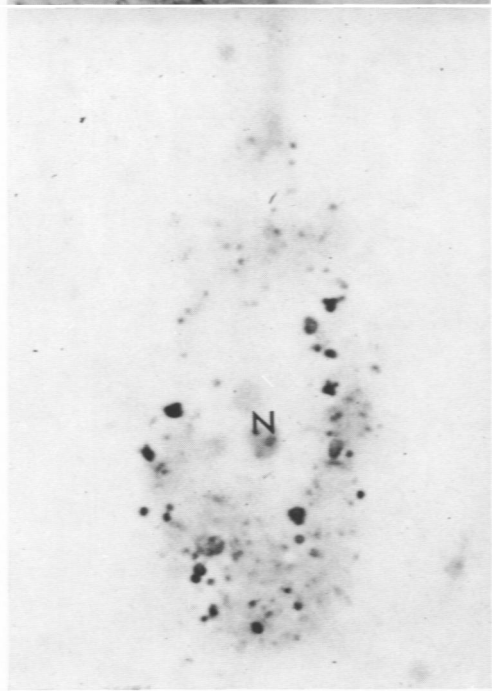

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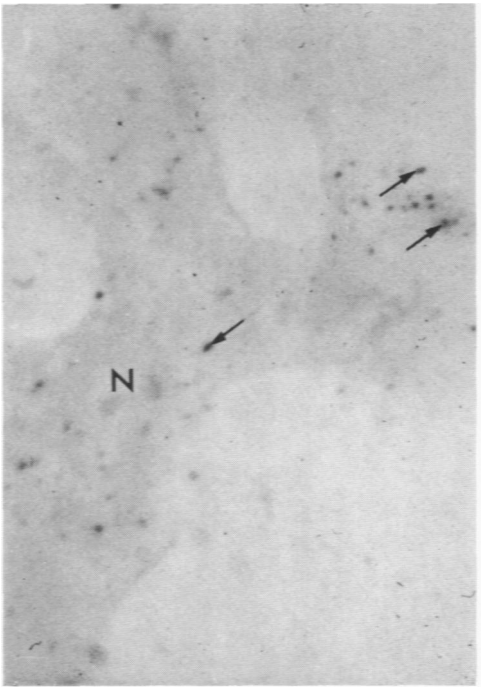

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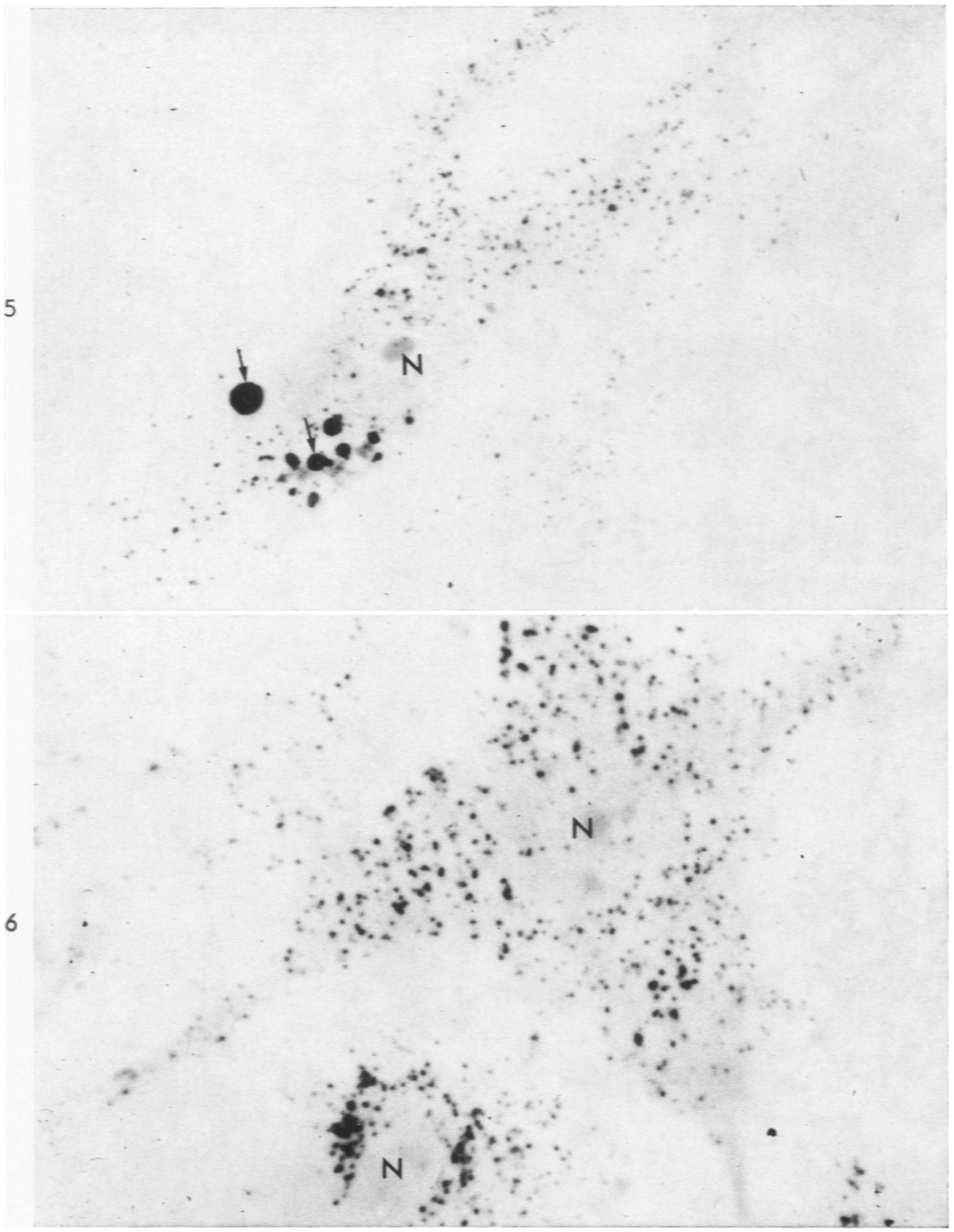

Fig. 5. Rheumatoid synovial cell. Incubated 45 min. for aryl sulfatase activity. Very large lysosomes resembling cytolosomes (? autophagic vacuoles) are seen (arrows) in these cells. Nucleus (N). × 1000. **Fig. 6.** Normal synovial cell. Incubated for aryl sulfatase activity at the same time and under the same conditions as preparation in Fig. 5. Although many lysosomes are stained, cytolosomes are not identified. Nucleus (N). × 1000.