

Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells

(fibroblast/macrophage/proteolytic enzymes/phagocytosis/lysozyme)

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Communicated by Jerome Cross, January 2, 1976

ABSTRACT We have studied cells dispersed with proteolytic enzymes from rheumatoid arthritic synovectomy specimens to determine the cell type(s) responsible for joint destruction. Initially 10-50% of these cells adhered to culture dishes within 24 hr and were of two main types: small, round cells and larger, stellate cells. During 1-4 days of culture, 5-25% had Fc receptors and 25-50% showed brisk phagocytosis. Daily production, per 10^6 cells, of collagenase (EC 3.4.24.3) (after trypsin pretreatment) was up to 70 μ g of collagen fibrils lysed per min at 37° (70 units), of prostaglandin (PGE_2), up to about 1200 ng, and of lysozyme, up to about 100 μ g. Under identical conditions of assay, fibroblasts grown from explants of synovium produced no detectable collagenase or lysozyme, and PGE_2 was only 2-4 ng. With the dispersed cell preparations, macrophage markers (Fc receptors and lysozyme) were undetectable after 4 days and PGE_2 decreased rapidly after about 7 more days. However, collagenase production continued for 3-25 weeks, and in some cultures, after cell passage. At these later stages, large, slow-growing stellate cells were predominant and could phagocytose carbon particles if incubated for >6-8 hr. Indomethacin (14 μ M) inhibited PGE_2 but stimulated collagenase production whereas dexamethasone (10 nM) inhibited both. Production of PGE_2 and collagenase in large amounts *in vitro* by these cells suggests that they may be involved in joint destruction *in vivo*. The precise origin of these synovial cells and the mechanisms responsible for the sustained production of collagenase at a high rate remain unidentified.

Rheumatoid arthritis (RA) is frequently accompanied by progressive destruction of joint structures, which is found predominantly in regions adjacent to masses of proliferating cells. Cultures of rheumatoid synovial tissue (containing, for example, proliferating lining cells, including those with macrophage properties, blood vessels, mononuclear cells, and fibroblasts) release collagenases capable of degrading undenatured collagen (1, 2) as well as prostaglandins which can accelerate bone resorption by osteoclasts (3, 4). It has not been determined previously which cells in these organ cultures are responsible for the release of such substances. Fibroblasts can be grown from human synovial explants, but we were previously unable to detect collagenase (EC 3.4.24.3) (2), and found only small amounts of prostaglandins (PGE_2) in these culture media (3). This contrasts with rabbit skin and synovial fibroblast cultures, which produce collagenase under some circumstances (5, 6). Recently, cultures of guinea pig macrophages have been shown to produce collagenase after stimulation by endotoxin (7) or by products of lymphocytes treated with phytohemagglutinin or specific antigens (8).

We reasoned that the methods of culturing cells from synovial explants favored the growth of cells, usually fibroblasts, that might not be responsible for the destructive effects of

proliferating synovium in RA. We therefore examined the heterogeneous population of cells from rheumatoid synovia for the ability to produce collagenase and PGE_2 , possible functional markers for certain of the potentially destructive cells in RA. Using proteolytic enzymes for dispersion of cells, we found that cells adherent to the surface of the culture vessels produce high quantities of collagenase and PGE_2 .

MATERIALS AND METHODS

Patients and Collection of Tissues. Sterile synovial tissue was obtained during therapeutic open joint surgery from patients with rheumatoid arthritis (RA). The diagnosis of RA was established according to criteria of the American Rheumatism Association (9).

Culture Methods. Within 2 hr after surgery, the sample was washed three times with Dulbecco's calcium- and magnesium-free phosphate-buffered saline (GIBCO), and pieces of the superficial layer of synovium of about 2 mm³ were cut and placed in Dulbecco's modification of Eagle's medium (GIBCO), supplemented with 100 units of penicillin and 100 μ g of streptomycin per ml (GIBCO), and containing 4 mg/ml of Clostridiopeptidase A (Worthington Biochemical CLS, 125-200 units/mg) sterilized through a 0.20 μ m filter (Nalge). The tissue fragments (0.5-1.0 g) were further divided with scissors in a 100 mm plastic petri dish (Falcon) and were then incubated for 3 or 4 hr in 20 ml of medium at 37° in a moist atmosphere of 5% carbon dioxide and 95% air.

The digest was well mixed many times by aspiration into and expulsion from a pasteur pipette. An equal volume of 0.05% trypsin and 0.02% EDTA in modified Puck's Saline A (GIBCO) was added and incubation continued for a further hour under the same conditions. The suspension was centrifuged 10 min at 400 \times g at room temperature and washed three times each with 40 ml of calcium- and magnesium-free phosphate-buffered saline. The pellet (1-2 ml) was suspended in modified Eagle's medium (20-40 ml) supplemented with 10% fetal bovine serum (Flow Laboratories), 100 units of penicillin, and 100 μ g of streptomycin per ml. Two milliliters of this final mixture were plated per 60 mm plastic petri dish (Falcon) or 1.0 ml was distributed on 12 mm coverglasses. The dishes were left undisturbed for 24 hr at 37°. They were then rinsed vigorously three times with phosphate-buffered saline, and 2 ml of fresh medium were added, the composition depending upon the study. Culture media from confluent fibroblast strains (3) derived from explants of synovial membrane and skin were also examined as described below.

Fc Receptors and Phagocytosis. Fc receptors were assayed by a modification of the method of Gordon and Cohn (10). Sheep erythrocytes in Alsever's solution (containing 5 \times 10⁹ cells per ml up to 4 weeks old) were washed three times with phosphate-buffered saline and then exposed to rabbit

Abbreviations: PGE_2 , prostaglandin E₂; RA, rheumatoid arthritis; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

anti-sheep erythrocyte antiserum (Cappel Laboratories) at a final dilution of 1:3200 in modified Eagle's medium. After a 20 min incubation at 37°, 1.0 ml of this solution was placed on coverslips. After 30 min at 37° the coverslips were gently washed in phosphate-buffered saline, fixed with absolute methanol, air dried, and stained with Giemsa.

India ink (Pelikan) or latex particles [polystyrene 1.01 μm diameter (Dow Diagnostics)] diluted 1:10 in phosphate-buffered saline were sterilized by autoclaving and 200 μl were added to 2 ml of modified Eagle's medium supplemented with 10% fetal calf serum and placed on coverslips. After various times at 37°, the coverslips were vigorously washed with phosphate-buffered saline, fixed, dried, and stained with Giemsa.

Assays. For assays of collagenase activity [^{14}C]glycine-labeled guinea pig skin collagen (specific activity 10,000 dpm/mg) was used (11). [^{14}C]Collagen was reconstituted as fibrils in 40 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2.7 mM CaCl_2 . One unit of collagenase activity is defined as the solubilization of 1 μg of reconstituted fibrils per min at 37°. Incubations were usually for 4 hr; all assays included a sample incubated with trypsin (100 $\mu\text{g}/\text{ml}$) to control for collagen denaturation.

In order to detect activity in samples containing 10% fetal bovine serum, 100 μl was added to 25 μl of trypsin-TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone) (Worthington TRTPCK) in 0.1 M Tris-HCl buffer, at pH 7.4 containing 5 mM CaCl_2 to give a final trypsin concentration of 200 $\mu\text{g}/\text{ml}$. After incubation for 10 min at 25°, 25 μl of soybean trypsin inhibitor (Worthington SI) in the same buffer was added to give a final concentration of 500 $\mu\text{g}/\text{ml}$. This reaction mixture was incubated for at least 10 min before 100 μl were removed for assay as described.

Viscometry, disc electrophoresis on polyacrylamide gels, and characterization of segment long spacing fragments were performed according to methods previously described (12). Lysozyme was assayed using suspensions of *Micrococcus lysodeikticus* in 1% agar (13); activity was expressed as μg equivalents of human lysozyme as a standard (gift of Dr. R. Canfield). Prostaglandins were assayed in culture media by radioimmunoassay (14), utilizing an antiserum with specificity towards prostaglandins E, A, and B. Prostaglandins from representative culture media were extracted with ethyl acetate and separated by thin-layer chromatography (15). The areas corresponding to PGE_2 and to PGA_2 and PGB_2 were identified by added tritiated marker compounds, and eluted from the chromatographic strips. Approximately 80% of the prostaglandins E + A + B was accounted for by PGE_2 and the remainder by PGB_2 . The method used does not distinguish between the one and two forms of either PGE_2 or PGB_2 . We have calculated the results in terms of PGE_2 based on our previous work demonstrating that rheumatoid synovial organ cultures produce PGE_2 and no detectable PGE_1 (16).

RESULTS

Cells from synovial tissue were readily dispersed using proteolytic enzymes as described. After 24 hr of incubation, the number of cells that adhered to the surface of culture vessels represented from about 10–50% of the total cells obtained from the tissue sample depending upon the initial character of the synovium. Of the cells that did not adhere during this period, 90–95% remained viable as judged by exclusion of Trypan blue; these were a mixed population, including lymphocytes, other leukocytes, and erythrocytes. The following

observations pertain to all synovial tissues cultured. In the early stages of culture, cells of varying morphology were present, many of which were relatively small (approximately 20 μm diameter) round cells with prominent well-stained nuclei, little cytoplasm, and few processes. With time, the morphology of the cell population changed so that fewer small cells were present, and after 10 days the cultures contained predominantly large cells with abundant cytoplasm, several processes, and large nuclei with 1 to 8 prominent nucleoli. The number of cells doubled approximately every 6–12 days, depending on the initial cell density, but the cells did not readily reach confluence; only large cells were present after many weeks of culture.

Attempts were made to maintain the cells in serum-free medium, as had been done previously with synovial organ culture (11) in order to detect collagenase not subjected to serum protease inhibitors (e.g., α_1 -antitrypsin and α_2 -macroglobulin). However, the synovial cells required serum and became nonviable if cultured in its absence in modified Eagle's medium for more than 2–3 days.

Macrophage Markers: Lysozyme Production, Fc Receptors, and Phagocytosis. Fc receptors, estimated by formation of rosettes with sensitized sheep erythrocytes, were present during the first 3 days in 5–25% of the cells in all cultures. Phagocytosis of carbon india ink particles was also observed in all cultures to a variable extent and in four samples was found in all cells during a 4 hr exposure. Both rosettes and intense phagocytosis were most evident in the smaller cells. In each of the three cultures in which lysozyme activity was assayed during the first 3 days, activity was produced in quantities of about 200–400 μg per 10^6 cells. The production of lysozyme and detectable Fc receptors declined rapidly with time and was undetectable in all cultures more than 7 days old. This correlated with the gradual disappearance of the smaller cells. Phagocytosis also diminished, but cells retained the ability to phagocytose latex or india ink particles for many weeks if the exposure to particles was increased to 6–24 hr, as has been noted for rabbit skin and synovial fibroblasts (5, 6).

Collagenase. In the absence of serum during the second to the fourth day of culture while some cells remained viable, collagenase could be detected in unconcentrated media at levels of 5–30 units/ 10^6 cells per day in six cultures from different patients. In the presence of serum, however, in which cells remained viable and able to replicate, no collagenase was detectable unless the medium was first incubated with trypsin. For example, in Fig. 1 is shown the collagenase activity for one culture after incubation with trypsin for 10 min at 25°, in final concentrations up to 200 $\mu\text{g}/\text{ml}$. In medium from that culture without serum the collagenase was already fully active so that treatment with trypsin produced little or no increase in activity. Indeed, incubation with trypsin at a final concentration of 1 mg/ml (not shown) caused a loss of up to 80% of the collagenase activity. When medium from the initially nonadherent cells was assayed for collagenase, small amounts were detected. However, with continued culture for 9 days, an additional fraction of these cells adhered to the vessel surface. Upon separation of the latter from those cells still nonadherent, only cultures of the adherent cells produced detectable collagenase, at levels about 5% of those in initially adherent cells.

Collagenase activity was detected in culture media from 16 out of 20 rheumatoid synovial samples examined. During the first week of culture the activity determined ranged from 20 to 70 units/ 10^6 cells per day. During the second

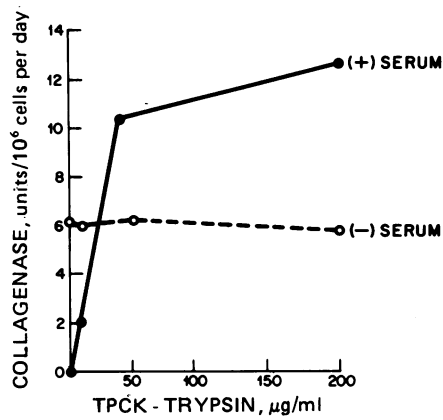


FIG. 1. Activation of collagenase in culture medium incubated with different final concentrations of TPCK-trypsin for 10 min at 25°. Synovial cells were cultured in modified Eagle's medium without (-) (O - - O) and with (+) 10% fetal bovine serum (● - ●).

week of culture the activity diminished to 3–15 units/ 10^6 cells per day, and during subsequent weeks became undetectable in most cultures. None of the cultures was passaged during the first 2 weeks of incubation. In four cultures of different samples the production of collagenase continued for several weeks at an apparently constant rate even after passage of the cells. In Fig. 2 are shown the results for one sample in which activity was measurable for 69 days. The maximum period in which activity was retained was 1 year in one sample.

Culture media with or without serum from five separate RA skin and synovial fibroblast strains derived by the explant technique (3) were also assayed after at least five passages for collagenase. Unconcentrated media or media concentrated up to 30-fold (Amicon-Minicon) with or without trypsin preincubation had levels of collagenase below the limit of detectability in our assay system (<0.02 unit/ 10^6 cells per day).

Characterization of Collagenase. Collagenase activity in media containing serum was not detected after gel filtration through Sephadex G-100 or G-50 unless the effluent fractions were preincubated with trypsin (latent collagenase). Culture media containing latent enzyme and treated with chymotrypsin yielded only 11–14% of the activity obtained with equal concentrations of trypsin.

The enzyme activities present in serum-free medium without trypsin activation and in serum-containing medium after trypsin activation both showed the typical characteristics of mammalian collagenase. Viscometric analysis of media from four separate cultures showed a time-dependent decline in specific viscosity to approximately 45% of the initial value, starting with collagen concentrations of approximately 1 mg/ml. In serum-containing media no change in specific viscosity occurred unless samples were preincubated with trypsin. Disc electrophoresis on polyacrylamide gels showed typical patterns of degradation products consistent with cleavage of the triple helix to produce the 25% TC^B and 75% TC^A fragments (17). Segment long spacing aggregates viewed by electron microscopy confirmed the presence of such reaction products, mostly as polymers but similar to those previously found with collagenases from synovial organ culture (11, 12) and other animal sources (17). Despite these observations, the possibility might still be considered that bacterial collagenase used for cell dispersion would contribute to the activity, particularly during the early stages of culture, even though EDTA was added to inactivate the bac-

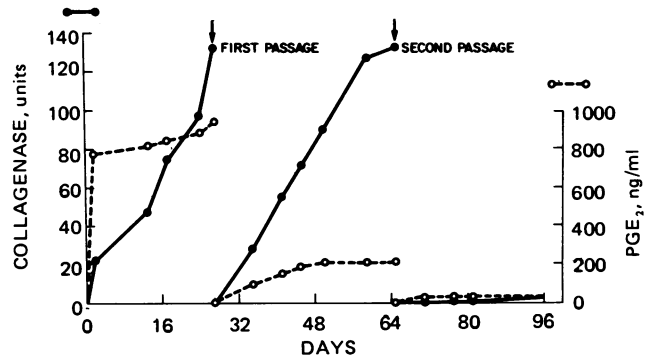


FIG. 2. Time course of collagenase and PGE₂ production from unstimulated, isolated, adherent rheumatoid synovial cells in monolayer culture. Cumulative totals of the collagenase and PGE₂ present in culture media from petri dishes (60 mm diameter) containing about 5.0×10^5 cells with changes of medium of 2 ml at each time point. Cells were passaged first at a dilution of 1 to 2 and then at a dilution of 1 to 8. Unconcentrated media activated by trypsin were assayed as described in the *text*. Although activity initially was very low in cells of the second passage, as shown here, subsequent cultures from the same sample planted at higher density continued to produce collagenase 8 months later.

terial enzyme and final dilution after washing was $>1 \times 10^{10}$. We noted that the products solubilized at 37° by medium from each of the nine early synovial cell cultures tested were $>90\%$ precipitated by 15% cold trichloroacetic acid. In contrast, bacterial collagenase in comparable activity in the fibril assay at 37° produced fragments of which $<10\%$ were precipitable in trichloroacetic acid. Furthermore in solution at 25°, below denaturation temperature of the collagen, multiple cleavage products were observed on gel electrophoresis using bacterial collagenase, but only bands corresponding to TC^A and TC^B were found with the synovial cell media. Finally, all media containing serum had no activity without trypsin activation, whereas similar levels of clostridial collagenase were only 50% inhibited by serum.

Prostaglandin Production. Synovial cells produced PGE₂ in high concentration during the early phase of culture. In cultures from the five different patients tested the amount of PGE₂ accumulated in the medium during the first 4–5 days was 1000–6000 ng per 10^6 cells (i.e., up to about 1200 ng/ 10^6 cells per day). In contrast to collagenase the rate of accumulation diminished rapidly with time, so that after several weeks the values were 10–100 ng per 10^6 cells per day (Fig. 2).

Drug Effects. Indomethacin, a known inhibitor of prostaglandin synthesis, at 5 $\mu\text{g/ml}$ (14 μM) reduced production of PGE₂ to $<0.1\%$, but caused a significant increase in the amount of collagenase present in culture media (Fig. 3). Dexamethasone at 0.01 and 1.0 μM markedly inhibited both collagenase and prostaglandin production (Fig. 4). Gold sodium thiomalate at 1 $\mu\text{g/ml}$ did not significantly affect production of collagenase (Fig. 3).

DISCUSSION

The major objective of this study was to culture rheumatoid synovial tissue in order to identify those cells responsible for the production of substances involved in joint destruction. We found that cultures of those cells that adhered to the vessel surface, although representing only a small proportion of the original population, were capable of producing large amounts of collagenase and PGE₂, as had been demonstrated previously for fragments of synovial tissue.

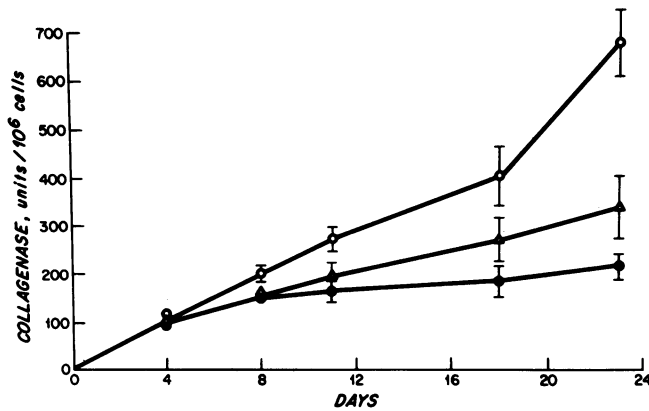


FIG. 3. Time course of production of collagenase from isolated adherent rheumatoid synovial cells in monolayer culture. Each point represents the cumulative totals of the collagenase (mean \pm SE) from three dishes (60 mm diameter) with medium changes of 2 ml per dish. Cultures with no additions of drug are indicated (\bullet); with addition of gold sodium thiomalate (1 μ g/ml) (Δ); with addition of indomethacin (14 μ M) (\circ). Cells were passaged using 0.05% trypsin, 0.02% EDTA.

The maximal rates of production of collagenase and PGE₂ are among the highest described for any cell type in culture. In our cells the collagenase activity was about 10 times the levels reported for "activated" rabbit synovial fibroblasts (5, 6), about 100 times those reported for human fibroblasts treated with cytochalasin B (18), and about 100 times those reported for macrophages activated by endotoxin (7), thioglycollate (19), or lymphocyte products (8).

Larger quantities of PGE₂ are also produced by these cells than by many other cell strains or established tumor cell lines reported in the literature. Confluent cell strains derived from normal and rheumatoid synovial explants after 4 to 6 passages produce 1–6 ng of PGE₂/10⁶ cells per day and human skin fibroblasts produce <2 ng of PGE₂/10⁶ cells per day (3), values approximately 0.1% of those reported here. Prostaglandin production by seven established mammalian tumor cell lines after several passages is estimated to be <10 ng of PGE₂/10⁶ cells per day (20, 21).

We are aware of only three reports of PGE₂ production by cell cultures as high (up to 1000 ng/10⁶ cells per day) as those described here for synovial cells. These include a mouse fibrosarcoma (20), a rabbit carcinoma (22), which caused hypercalcemia attributed to the large quantities of PGE₂ released, and BHK fibroblasts transformed by polyoma virus (23).

The cells responsible for the activity in our study have not been identified. In the early stages of culture, several cell types are present and the production of collagenase and PGE₂ is high. As the cultures age and the cells become more homogeneous, PGE₂ levels fall off more rapidly than collagenase. Since macrophages can produce collagenase (7, 8, 19), the possibility arises that the synovial A cells, which resemble macrophages (24), are the source of collagenase and prostaglandin. However, markers for macrophages (10, 25, 26), namely, Fc receptors, lysozyme production, and intense phagocytic activity, become undetectable within a few days of culture, suggesting that the macrophage-like cells lose some of their specific characteristics or do not survive for prolonged periods. This contrasts with the ability of macrophages from other sources to survive for months in culture and to retain these markers (25, 26). The cells possessing macrophage markers might be a source of the PGE₂ since its production is greatest while they are present. However, in

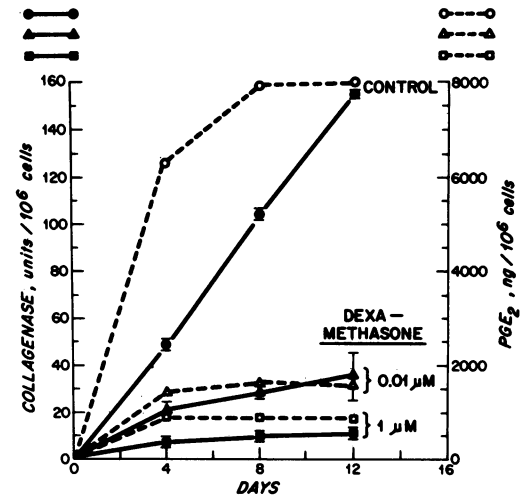


FIG. 4. Time course of production of collagenase (—) and PGE₂ (---) from isolated adherent rheumatoid synovial cells in monolayer culture. Each point represents the cumulative totals of the collagenase and the PGE₂ (mean \pm SE) from three dishes (60 mm) with changes of medium of 2 ml per dish. Cultures with no addition of drug are indicated (\bullet , collagenase; \circ , PGE₂); addition of dexamethasone (0.01 μ M) (\blacktriangle , collagenase; \triangle , PGE₂); addition of dexamethasone (1 μ M) (\blacksquare , collagenase; \square , PGE₂).

studies not shown, we found levels of PGE₂/10⁶ cells per day in two primary human blood monocyte cultures to be 30 and 96 ng, and <5 ng in mouse peritoneal macrophage cultures. Collagenase was undetectable in these cultures.

The synovial cells that survive and replicate in our cultures differ from cells obtained by outgrowth from synovial explants and from skin fibroblasts in several respects, which include morphology, rate of replication, serum dependence, phagocytic potential, and collagenase production. Previous studies of cells from rheumatoid synovial tissue have been restricted to those strains obtained by the explant technique (27) or from cultures of synovial fluids (28). Although such cells differ from normal synovial fibroblasts, for example, in glycolytic rates and hyaluronate production (27), the difference in collagenase production between our synovial cells and explant cells under conditions described is many times greater (>1000-fold).

The collagenase produced by the synovial cells is typical of other animal collagenases, and the bacterial collagenase used during the cell isolation was clearly shown not to contribute detectable activity to the culture media. The ability to detect synovial collagenase after incubation of cells in medium containing serum is unusual since most studies have utilized serum-free medium in order to obviate inhibition of collagenase by factors such as α_2 -macroglobulin and α_1 -antitrypsin. Collagenase activity was revealed only after brief exposure to trypsin, as has been observed by others for bone (29) and skin enzyme (30). There are several possible mechanisms by which trypsin could activate the enzyme. In preliminary studies latent collagenase activity was retarded on columns of Sephadex G-75, and well separated from the fractions containing α_2 -macroglobulin, indicating that inhibition by α_2 -macroglobulin is unlikely to account for the latency. The presence of inhibitors of lower molecular weight (31) could explain the latency as could the existence of a zymogen (17, 29).

The production of collagenase and PGE₂ by synovial cells is of potential importance in relation to destructive processes in arthritis. Collagenase, together with other tissue proteases, may play a major role in the degradation of articular carti-

lage and other joint structures (1, 2). Prostaglandin E₂, at concentrations found in the cell cultures, may have several effects, one of which may be to promote resorption of bone (3, 4). Whereas inhibition of prostaglandin production by indomethacin was anticipated, stimulation of the collagenase was unexpected and suggests that collagenase production is not a result of cell stimulation by PGE₂. However, dexamethasone inhibits both PGE₂ and collagenase production at therapeutic concentrations (10 nM). The present findings contrast with observations that much higher concentrations of dexamethasone (1 mM) increase collagenase in cultures of corneal fibroblasts (32), but confirm those of Koob *et al.* (33), who found that 10 nM dexamethasone inhibited collagenase production in skin, uterus, and synovial organ cultures.

We are uncertain whether collagenase production is specific for cells from RA synovium as compared with normal or that from other forms of joint disease, since collagenase production by organ cultures is also not specific for RA (34). However, the cells studied here are probably those responsible for collagenase production by the RA organ cultures. Although it is possible that the methods used for isolation of the cells, namely, digestion with bacterial collagenase and trypsin, activates the cells, this seems unlikely to be the sole mechanism since organ cultures of rheumatoid synovia also produce collagenase and prostaglandins. Many possible mechanisms of stimulation exist. These include infection by viruses or mycoplasmas, response to endotoxin, stimulation by phagocytosis or endocytosis (perhaps of immune complexes), or as a result of cell activation by products of other cells such as lymphocytes. These possibilities should be susceptible to experimental analysis.

We are grateful to P. Rogers, W. Karge, M. McGuire, and D. Bastian for technical assistance and to R. Johnson for preparation of the manuscript. We also thank the orthopedic surgeons who provided us with tissue, particularly Drs. W. Harris, W. Jones, T. Potter, C. Sledge, and R. Smith, and Dr. D. DiBona for preparation of electronmicrographs. Supported by Grants AM 03564, AM 0567, and AM 04501 and by a grant from the Massachusetts Chapter of the Arthritis Chapter of the Arthritis Foundation. J.-M.D. has been supported by grants from Janggen-Pöhn-Stiftung and Fonds National Suisse de Recherche Scientifique. This is Publication no. 693 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities.

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