Collagenase Production by Rheumatoid Synovial Cells: Stimulation by a Human Lymphocyte Factor

Abstract. *Human peripheral blood lymphocytes incubated in culture for 1 to 3 days at 37°C, but not at 4°C, release a soluble factor which can stimulate, up to 400-fold, collagenase production by isolated, adherent, rheumatoid synovial cells. Production of lymphocyte factor is enhanced by phytohemagglutinin or concanavalin A. By gel filtration the factor has an apparent molecular weight of about 12,000.*

Augmented production of collagenase by hypercellular synovial tissue may be an important cause of the destruction of joint structures which characterizes rheumatoid arthritis (*1, 2*). We have devised methods (*3*) for isolating rheumatoid synovial cells that adhere to culture vessels and produce typical mammalian collagenase for prolonged periods (weeks to more than 1 year). These cells are large, slow-growing, and morphologically distinct from fibroblasts, and lack macrophage markers. In searching for natural and synthetic agents that might influence collagenase production by synovial cells, we have examined lymphocytes as a potential source of such material. Lymphocytes are present in rheumatoid subsynovial infiltrations (*4*), and immunological disturbances are features of rheumatoid arthritis (*5*). Recently, several lymphokine-like substances, such as a migration inhibitory factor (MIF) and mitogenic factors, have been described in synovial fluid and in the supernatants of synovial tissue cultures from patients with rheumatoid arthritis (*6*). In addition, products released by guinea pig lymphocytes stimulated with concanavalin A or specific antigens have been reported to increase collagenase production by guinea pig macrophages in culture (*7*). In this report we describe a factor released by human lymphocytes incubated in culture which markedly stimulates collagenase production by rheumatoid synovial cells.

Heparinized venous blood from normal persons or patients with classical rheumatoid arthritis was used as a source of lymphocytes which were separated on gradients of sodium metrizoate and Ficoll (Lymphoprep, Nyegaard, Oslo) (*8*). The mononuclear cells (2×10^6 cells per milliliter) were cultured at 37°C in an atmosphere of 5 percent CO_2 and 95 percent air, in 6-cm-diameter polystyrene petri dishes (Falcon) in Dulbecco's minimum essential medium (MEM) containing 10 percent fetal calf serum (FCS; Microbiological Associates). After 24 hours the nonadherent lymphocytes were separated from the adherent monocytes, centrifuged, resuspended in fresh MEM and 10 percent FCS, and incubated for a further 3 days.

The lymphocyte-free supernatant medium (LM) prepared by centrifugation (3000*g* for 10 minutes) was used as a source of the stimulating factor. Collagenase activity was undetectable in all LM. To test for stimulating activity, this LM was diluted with MEM and 10 percent FCS (1:6 to 1:40, depending upon the experiment). These LM were incubated with adherent rheumatoid synovial cells in 6-cm-diameter polystyrene petri dishes or in trays (Costar) containing 24 wells each of 16 mm in diameter. After 1 to 3 days, collagenase activity was assayed in the medium after prior treatment with trypsin (*3*); ^{14}C-labeled reconstituted guinea pig skin collagen fibrils were used as substrate (*9*). Our previous studies had shown that, although collagenase activity was detectable without trypsin treatment in serum-free media from synovial cells, the inclusion of serum in the media, which was essential for prolonged survival of the synovial cells, rendered the collagenase undetectable unless the medium was first incubated with trypsin (*3*). One unit of collagenase activity represents the lysis of 1 μg of collagen per minute.

The addition of LM increased the collagenase released by rheumatoid synovial cells from each of ten different patients. The stimulation was approximately two- to threefold for synovial cells tested soon after isolation when basal activity was still high (*3*). After several months in culture or after several passages of the synovial cells, when spontaneous, basal production of collagenase had fallen to low levels, the relative stimulation by LM was much greater (up to 400-fold). In some synovial cell cultures incubated with LM, collagenase levels reached those found in freshly isolated synovial cells. Examples of typical responses are shown in Fig. 1 and Table 1. Each of the 28 lymphocyte preparations

Table 1. Collagenase production by synovial cells after exposure to LM. In each different experiment the synovial cells were derived from a different subject: lymphocytes used in experiment 1 were from a different normal subject from those used in experiments 2 and 3 (same normal subject). Adherent rheumatoid synovial cells after one passage were plated into wells (experiment 1, 6×10^4 cells per well; experiments 2 and 3, 1×10^4 cells per well) and incubated for 2 days (experiment 1) and 5 days (experiments 2 and 3) in MEM, with 10 percent FCS. After this time the medium was removed and replaced with diluted (experiment 1, 1:6; experiments 2 and 3, 1:10) fresh MEM and 10 percent FCS. Incubation of the synovial cells was continued for an additional 41 hours and collagenase released into the medium assayed. Equal portions of the lymphocytes previously incubated for 3 days at 37°C (experiment 1, 2×10^6 cells per milliliter; experiments 2 and 3, 1×10^6 cells per milliliter) as shown above were removed for the last 16 hours of incubation and [^{3}H]thymidine was added (4×10^6 count/min), to assess the mitogenic effects of phytohemagglutinin (PHA) or concanavalin A (Con A) by isolation of [^{3}H]DNA on glass fiber strips after filtration. All values represent the mean ± standard error for three wells in experiment 1 and four wells in experiments 2 and 3.

Treatment of LM		[^{3}H]Thymidine incorporation by lymphocytes (count/min per well)	Collagenase production by synovial cells (units per well)
Lymphocytes (3 days)	Mitogen (5 μg/ml)		
		Experiment 1	
−	None		0.20 ± 0.01
+	None	1,082 ± 103	3.67 ± 0.30
+	PHA	16,341 ± 1,993	5.15 ± 0.77
−	PHA		0.11 ± 0.01
+*	None	1,168 ± 103	4.28 ± 0.19
		Experiment 2	
−	None		Undetectable
+	None	6,156 ± 329	Undetectable
+	PHA	19,339 ± 1,757	10.91 ± 1.10
−	PHA		Undetectable
+	Con A	6,769 ± 149	16.62 ± 0.93
−	Con A		Undetectable
		Experiment 3	
−	None		1.92 ± 0.70
+	None	6,156 ± 329	24.94 ± 1.99
+	PHA	19,339 ± 1,757	26.95 ± 3.05
−	PHA		1.84 ± 0.53
+	Con A	6,769 ± 149	29.56 ± 4.25
−	Con A		1.12 ± 0.50

*This sample contained 10 percent heat-inactivated autologous human serum, whereas all the other samples contained 10 percent FCS.

tested (from four control and three rheumatoid subjects) stimulated the activity of synovial cells prepared by proteolytic dispersion. Collagenase production was increased within the first 38 hours of exposure to LM (Fig. 1). The rate of collagenase production then declined after 3 days and in other experiments, not shown, reached baseline levels within 10 to 20 days after the removal of the LM and could be restimulated by a second exposure to LM. The continued response of synovial cells to LM seemed to depend on cell density as well as on the passage number and age of the cultures. The collagenase from stimulated synovial cultures yielded cleavage products of collagen which were identical to those obtained with other typical mammalian collagenases, as determined by polyacrylamide disc gel electrophoresis (data not shown) (*1*).

Figure 1 also shows that increasing the time of incubation of lymphocytes from 1 to 3 days resulted in increased stimulation of collagenase by synovial cells exposed to these LM. The stimulating factor produced by lymphocytes was not present in supernatants of freshly isolated lymphocytes lysed by repeated freezing and thawing or in cultures of lymphocytes incubated at 4°C. It was possible, with some synovial cell cultures, to establish a pattern of dose-response to LM. In the experiment depicted in Fig. 2 there was a linear increase in collagenase produced which was proportional to the logarithm of the volume of LM.

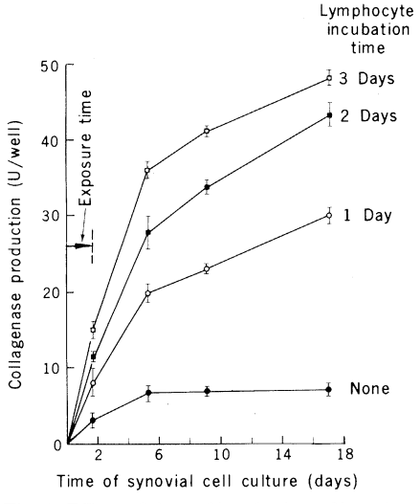

Fig. 1. Effect of time of incubation of lymphocytes on the production of collagenase-stimulating activity (measured as units per well). Lymphocytes (2×10^6 cells per milliliter) from a normal subject were incubated for 1, 2, or 3 days at 37°C. The cell-free medium was diluted 1:6 with fresh MEM and 10 percent FCS, and exposed for 38 hours to adherent rheumatoid synovial cells, plated 18 days previously in multiwell trays (1×10^5 cells per well). Synovial cell media were replaced with fresh MEM and 10 percent FCS, in all wells at the times shown. The points represent the mean ± standard error of collagenase measured in media from three wells, plotted to show the cumulative production of collagenase throughout the experiment. Spontaneous production of collagenase was measured in wells not exposed to LM (bottom curve).

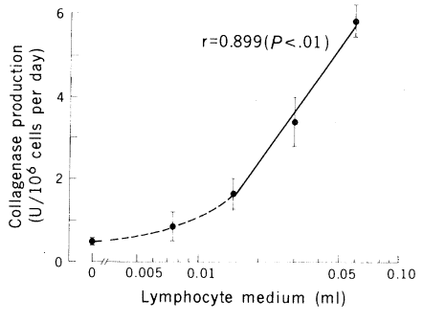

Fig. 2. Production of collagenase by synovial cells in response to the logarithm of the volume of added LM. Lymphocytes (1×10^6 cells per milliliter) from a normal subject were incubated for 3 days at 37°C. The cell-free medium in volumes indicated was added to cultures of adherent rheumatoid synovial cells plated 5 days previously, in multiwell trays (1×10^5 cells per well; total volume 0.3 ml) and incubation continued for 41 hours at 37°C. The points represent the mean ± S.E. of collagenase measured in media from each of four wells. The correlation coefficient (r) is calculated for the three points connected by the solid line.

The stimulating factor is produced by lymphocytes rather than by monocytes. The cells which adhered to culture vessels during the first 24 hours of incubation and remained after washing, and which were then incubated for an additional 3 days, produced no detectable stimulating activity. The possibility that some early interaction occurs between monocytes and lymphocytes cannot be excluded and we cannot say whether B or T lymphocytes, or both, are involved. Collagenase-stimulating activity was not present in media exposed to comparable numbers of normal and SV40-transformed fibroblasts [SV clone 80 (*10*)] and a lymphoblast line transformed by Epstein-Barr virus.

The previous observations indicated that intact cell metabolism and the incubation of lymphocytes in culture are necessary for the spontaneous production of the collagenase-stimulating factor. Spontaneous production of an accepted lymphokine (MIF) has been described in human blood mononuclear cells cultured in autologous serum alone (*11*). Heat-inactivated (56°C for 45 minutes) autologous human serum (10 percent) and FCS (10 percent) could substitute for the nonheated FCS usually used in our lymphocyte cultures (Table 1). When spontaneous production of the stimulating factor was high and the synovial cells relatively sensitive, no significant effect of phytohemagglutinin on the production of the factor was observed (experiment 1, Table 1). The stimulating effect of the lectins on production of the factor was revealed either by diluting the LM or by using less sensitive cells to assay the response at low dilution (experiments 2 and 3, Table 1). When older, less sensitive synovial cells were tested for their response, significant increase in stimulating factor production was found in cultures of normal lymphocytes incubated with either phytohemagglutinin or concanavalin A (experiment 2, Table 1). The same dilution of LM used in experiment 2 (Table 1) would cause the maximum stimulation of another culture of synovial cells in the absence of lectin (experiment 3, Table 1). Both normal and rheumatoid lymphocytes produced stimulating factor spontaneously.

The stimulating activity was stable in MEM and 10 percent FCS for at least several days at room temperature and for months at −30°C; it was not inactivated by heat (56°C for 30 minutes). When culture media from unstimulated lymphocytes were subjected to gel filtration on calibrated columns of Ultrogel AcA54 (Fig. 3), collagenase-stimulating factor was eluted as a single sharp peak, just be-

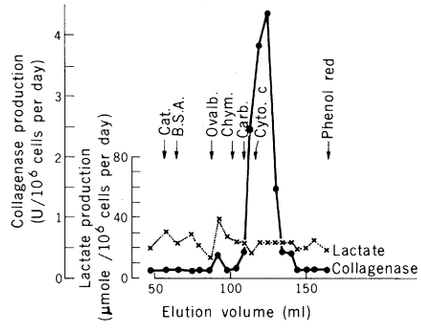

Fig. 3. Effect on synovial cells of lymphocyte culture medium after gel filtration. Lymphocytes (2×10^6 cells per milliliter) from a patient with rheumatoid arthritis were incubated for 3 days in MEM and 10 percent FCS. The cell-free medium (3 ml) was applied to a calibrated column (36.5 by 2.5 cm) of Ultrogel AcA54 (LKB Instruments) equilibrated and eluted with MEM without FCS. Fetal calf serum was added to each fraction to bring its final concentration to 10 percent. These fractions were then incubated with adherent rheumatoid synovial cells (1×10^5 cells per well) in the fourth passage which had been plated 5 days previously. After a further 3 days the media were assayed for collagenase (solid line). Lactate (dotted line) was assayed in neutralized perchloric acid extracts (*14*). Substances used for molecular weight calibration were eluted in positions shown. Abbreviations; *Cat.*, catalase; *B.S.A.*, bovine serum albumin; *Ovalb.*, ovalbumin; *Chym.*, chymotrypsinogen; *Carb.*, carbonic anhydrase; *Cyto. c*, cytochrome c.

hind the cytochrome c marker, suggesting a molecular weight of about 12,000. The behavior on gel filtration of media from lymphocytes incubated in the presence of lectins was not determined. Crude LM or activity partially purified by gel filtration did not increase the proliferation of synovial cells over controls as judged by cell counting or by uptake of [^{3}H]thymidine (not shown), nor did it stimulate lactate production (Fig. 3), suggesting an effect different from the so-called connective tissue–activating peptide which characteristically stimulates glycolysis in its target cells (*12*). The synthesis of lysozyme, a characteristic product of macrophages (*13*), was also not stimulated by the factor, providing additional evidence that the responsive synovial cells are distinct from typical macrophages (*3*). Furthermore, we could not detect collagenase activity in cultures of human peripheral blood monocytes exposed to lymphocyte media. The levels of collagenase in guinea pig macrophages stimulated by lymphocyte products as reported by Wahl *et al.* (*7*) are calculated to be less than 0.1 percent of those of the stimulated human synovial cells in the present study.

The biological significance and specificity of the production and effects of this lymphocyte factor remain to be determined. Lymphocytes are abundant in the subsynovial region in rheumatoid synovitis, and the production of this stimulating factor might serve as a cell-to-cell mediator acting between lymphocytes and susceptible target cells in the proliferative synovial lining layer. If the factor can stimulate collagenase in vivo, it may have an important role in regulating connective tissue turnover, and may be responsible for some of the deleterious effects on joint structures in rheumatoid arthritis and connective tissue resorption in a variety of other inflammatory disorders.

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Localization of Cyclic GMP and Cyclic AMP in Cardiac and Skeletal Muscle: Immunocytochemical Demonstration

Abstract. *When rat cardiac and skeletal muscle are explored by immunocytochemical procedures designed to show sites of localization of adenosine 3',5'-monophosphate (cyclic AMP) and guanosine 3',5'-monophosphate (cyclic GMP), distinct staining patterns for the two nucleotides are seen. Antibody to cyclic AMP is found in the area of the sarcoplasmic reticulum, while antibody to cyclic GMP is found with a periodic distribution corresponding to that of the A band. This suggests a role for cyclic GMP in the regulation of myosin.*

The regulation of calcium concentration by the membranes of the sarcoplasmic reticulum is important in coupling excitation and contraction in muscle. Evidence suggests that adenosine 3',5'-monophosphate (cyclic AMP) is involved in the sequestration of calcium by the sarcoplasmic reticulum (*1*). Cyclic AMP stimulates the activity of protein kinase and increases calcium uptake by enriched preparations of fragmented sarcoplasmic reticulum isolated from cardiac and fast skeletal muscle (*1*). Calcium uptake by the sarcoplasmic reticulum is associated with relaxation of both cardiac and skeletal muscle (*2*).

Changes in the intracellular concentrations of cyclic AMP and cyclic GMP have been observed in each contraction cycle in heart (*3*). In addition, cyclic GMP levels increase in cardiac muscle after stimulation with acetylcholine (*4*). However, the role of cyclic GMP in the control of contraction is not understood. In a number of tissues in vitro the increased concentration of cyclic GMP observed after the addition of acetylcholine and other agents depends upon the presence of extracellular calcium (*5*). This suggests that calcium fluxes participate in the control of intracellular cyclic GMP concentrations. These and other experiments have led to the postulate that cyclic GMP might participate in the feedback regulation of calcium transport across membranes (*6*).

To investigate the roles of both cyclic nucleotides in cardiac and skeletal muscle, we have used an immunocytochemical technique that reveals sites of localization of cyclic AMP and cyclic GMP in specific cell types and in a variety of tissues (*7*). We have found that in a number of rat tissues cyclic GMP is predominantly located in the plasma membrane area and in the nucleus, whereas cyclic AMP is often found in cytoplasmic elements and in the plasma and nuclear membrane areas. Since cyclic nucleotide immunocytochemistry utilizes unfixed tissues, and free cyclic nucleotide might be lost during the staining procedure, sites of localization of these cyclic nucleotides appear to indicate sites of binding or receptor proteins.

Cardiac and skeletal muscle were obtained from rats killed by cervical dislocation. Skeletal muscle was taken from the lower part of either the fore or hind limbs and the skin quickly dissected away. Tissues were immersed in OCT compound (optimal cutting temperature; Miles, Elkhart, Indiana) and frozen in an aluminum container in a mixture of acetone and solid CO_2. Tissues were frozen within 1 minute after removal, and were later cut into sections, 3 to 4 μm thick, with a cryostat. The sites of localization of both nucleotides were determined by an indirect immunofluorescent technique (*7*) for which we used rabbit immunoglobulin (Ig) specific to either cyclic AMP or to cyclic GMP, prepared by the method of Steiner *et al.* (*8*). Air-dried slides were treated sequentially as described previously (*7*). To test the specificity of the procedure, we showed that similarly prepared Ig fractions from unimmunized rabbits failed to produce significant staining. In addition, passage of the specific