Studies on Purified Rheumatoid Synovial Collagenase In Vitro and In Vivo

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ABSTRACT Rheumatoid synovial collagenase obtained from culture medium can be separated by Sephadex gel filtration into two peaks of enzyme activity. These have been designated as fast-moving and slow-moving rheumatoid synovial collagenases on the basis of their electrophoretic mobility on polyacrylamide gels. The slow-moving rheumatoid synovial collagenase has been highly purified by affinity chromatography on collagen conjugated to Sepharose and used to prepare a monospecific anti-synovial collagenase antiserum.

The antiserum against rheumatoid synovial collagenase has permitted the demonstration of immunoreactive collagenase in extracts of rheumatoid synovial tissue that have no detectable enzymatic activity. Collagenase has also been detected immunologically in enzymatically inactive culture medium from the first 24 hr of culture. Recovery of collagenase activity appears to be related to the chromatographic separation of the enzyme from serum antiproteases. The demonstration of collagenase in vivo in rheumatoid synovium adds further support for the concept that the enzyme is present in tissue at levels that are of significance in the pathogenesis of rheumatoid arthritis.

In addition, rheumatoid synovial collagenase and human skin collagenase show complete immunologic identity when reacted with monospecific antiserum prepared against either of these purified enzymes, indicating that organ specificity between these two human collagenases is unlikely.

INTRODUCTION

A possible mechanism for the destruction of cartilage seen in rheumatoid arthritis was suggested by Evanson, Jeffrey, and Krane (1, 2) with the demonstration that rheumatoid synovium in tissue culture synthesizes and releases into the medium a collagenase capable of specifically degrading native collagen at neutral pH and 37°C. Although the enzyme could be found in culture medium of synovial explants from certain other inflammatory diseases (3), no collagenase was elaborated by normal synovial tissue.

Further evidence that collagenase is involved in the pathogenesis of rheumatoid arthritis came with the detection of collagenase activity in the synovial fluid from some patients with rheumatoid arthritis (4) and the demonstration that rheumatoid synovium is capable of degrading the collagen of whole cartilage (5). In addition, collagenase activity in rheumatoid arthritis is related to the degree of both local and systemic disease activity (3, 6). These findings indicate that collagenase derived from synovial tissue can produce the destruction of cartilage seen in rheumatoid arthritis.

The preparation of an antiserum against a purified human skin collagenase has permitted certain relevant observations to be made concerning synovial collagenase (7). The major finding is that immunologic cross-reactivity exists between human skin and rheumatoid synovial collagenases (7, 8). In spite of this cross-reactivity, minor variations in antigenic structure, indicative of organ specificity between these human collagenases, could not be investigated until an antiserum prepared against a purified synovial enzyme was available.

Utilizing antiserum to human skin collagenase (9) it has also been shown that collagenase is present in human skin extracts that have no detectable enzymatic activity. Collagenase activity can be recovered from skin extracts by separation of the enzyme from the serum antiproteases, α_1-antitrypsin, and α_2-macroglobulin, which are known to inhibit collagenase (10). It was also demonstrated that the characteristic delay of 24–48 hr in the appearance of human skin collagenase in the culture medium was related to the inhibition of collagenase activity by serum alpha globulins present in the medium shortly after the tissue explants were initially placed in culture.

Received for publication 26 March 1971 and in revised form 24 May 1971.

A similar delay in the appearance of synovial collagenase into culture medium has also been observed (2). In addition, a nondialyzable inhibitor of the enzyme was found to be present in the culture medium of synovial explants only during the first 2 days of culture (11). This inhibitor has not been characterized further and its possible relationship to serum antiproteases is unknown.

In view of the potential significance of synovial collagenase in the pathogenesis of rheumatoid arthritis, a number of important questions arise. (*a*) Can synovial collagenase be detected in rheumatoid synovium in vivo? (*b*) Is the lag in the appearance of active synovial collagenase into the medium of cultures of synovial explants related to its inhibition by serum antiproteases? (*c*) Does immunologic organ specificity exist between human skin and synovial collagenases?

Using a monospecific antiserum, prepared against synovial collagenase purified from the culture medium of rheumatoid synovial explants, we have been able to demonstrate the presence of the synovial enzyme both in vivo and in vitro under circumstances in which serum antiproteases may make its detection impossible. In addition, it has been possible to establish more precisely the relationship of human synovial collagenase to human skin collagenase.

METHODS

Culture techniques. Synovial tissue from patients with rheumatoid arthritis was obtained at surgery, trimmed of fat, and cut into 3-mm^3 pieces. Explants were cultured in disposable flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) in Dulbecco's Modified Eagle's medium in an atmosphere of O_2-CO_2 (95 : 5, v/v) as described by Evanson et al. (1, 2). Culture medium was harvested daily for 10 days and examined for collagenase activity as well as for immunologic evidence of collagenase, α_2-macroglobulin, and α_1-antitrypsin. Media having collagenase activity were pooled, dialyzed against several changes of distilled water at 4°C, and lyophilized. Crude enzyme powder was stored at −20°C. Medium from the first 24 hr of culture was dialyzed and lyophilized separately from that of the remaining days.

Enzyme purification. Crude synovial collagenase powder was dissolved in 0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$ to a concentration of approximately 20 mg protein per ml. Ammonium sulfate fractionation was carried out at pH 7.0 and 0°C to a final saturation of 60%. The 60% precipitate was then dissolved in 0.05 M Tris-HCl (pH 7.5) with 0.005 M $CaCl_2$ and dialyzed against large volumes of the same buffer.

Gel filtration was performed using reverse flow at 4°C on a column 1.2 × 100 cm of Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), equilibrated with 0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$ and 0.2 M NaCl in a manner similar to that described by Harris, DiBona, and Krane (4). Fractions having collagenase activity were pooled, dialyzed against 0.05 M Tris-HCl (pH 7.5) with 0.005 M $CaCl_2$, and concentrated by pressure dialysis. The broad peak of enzyme activity obtained from Sephadex G-150 was further fractionated by gel filtration on a column (0.9 × 60 cm) of Sephadex G-75 which has been equilibrated with the same buffer in a manner similar to that used for human skin collagenase (7).

Affinity chromatography. Collagen was coupled to Sepharose 4B (Pharmacia Fine Chemicals Inc.) according to the method described for other ligands by Cuatrecasas, Wilchek, and Anfinsen (12, 13). 25 ml of cyanogen bromide–activated Sepharose 4B was suspended in 50 ml of cold 0.2 M $NaHCO_3$ (pH 9.0), and 75 mg of purified native guinea pig skin collagen in 25 ml of 0.4 M NaCl was added immediately. The mixture was stirred gently for 18 hr at 4°C, washed with water, and equilibrated with 0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$. Using these conditions, approximately 80% of the collagen was coupled to the Sepharose.[1]

Affinity chromatography was performed on a column (1.2 × 1.5 cm) of collagen-Sepharose, equilibrated with 0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$ at 4°C. 5–10 mg of partially purified synovial collagenase was applied to the column in approximately 2 ml of the same buffer. The column was washed with starting buffer until the absorbance at 280 mμ returned to base line. Collagenase was then eluted with 0.05 M Tris-HCl, 0.005 M $CaCl_2$ containing 1.0 M NaCl. The eluent peak was dialyzed against several changes of distilled water and lyophilized. The enzyme powder was reconstituted in 0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$ and assayed for collagenase activity. Unbound material from the exclusion peak was handled in a similar manner and was subjected to rechromatography on collagen-Sepharose until no enzyme activity remained.

Enzyme purification was monitored using polyacrylamide gel electrophoresis on 12.5% gels (14). In all experiments a Tris-glycine (pH 8.5) buffer was used (15).

Day 1 cultures. Approximately 135 mg of crude powder from day 1 cultures, which contained no detectable collagenase activity, was applied in 0.05 M Tris-HCl, 0.005 M $CaCl_2$, pH 7.5, to a 2.5 × 100 cm column of Sephadex G-150. Gel filtration was performed using reverse flow at 4°C in a buffer of 0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$ and 0.2 M NaCl. Eluent fractions were assayed for collagenase activity and examined for immunologic evidence of collagenase, α_2-macroglobulin, and α_1-antitrypsin.

Human skin collagenase. Human skin collagenase was obtained from cultures of human skin explants (16). The electrophoretically pure fast-moving human skin collagenase was prepared as previously described (7).

Antiserum preparations. Antiserum to synovial collagenase, purified by affinity chromatography, was prepared in rabbits, and the gamma globulin fraction was obtained in a manner similar to that previously described for preparation of anti-human skin collagenase antibodies (7).

Anti-human α_1-antitrypsin was obtained from Hoechst (Beringwerke) Pharmaceutical Co., Kansas City, Mo. and anti-human α_2-macroglobulin was purchased from Hyland Laboratories, Los Angeles, Calif.

Immunologic procedures. Gel diffusion was performed on 0.75% Ionagar, buffered with 0.05 M Tris-HCl (pH 7.5) containing 0.15 M NaCl (17). Quantitative precipitin reactions were carried out by adding increasing amounts of synovial collagenase to 0.1 ml of anti-synovial collagenase gamma globulin and adjusting the final volume to 0.25 ml

[1] Bauer, E. A., J. J. Jeffrey, and A. Z. Eisen. 1971. Preparation of three vertebrate collagenases in pure form. *Biochem. Biophys. Res. Commun.* In press.

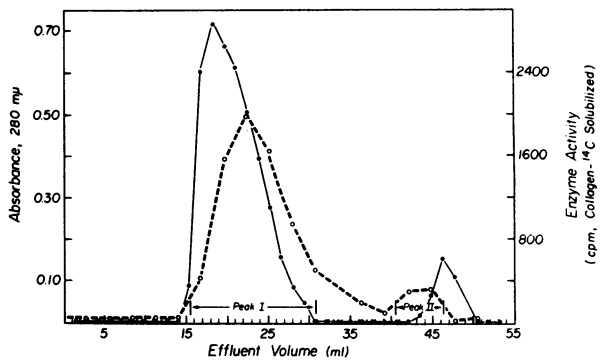

FIGURE 1 Gel filtration of synovial collagenase on Sephadex G-75. A sample of 5.2 mg of partially purified enzyme protein (see Methods) was applied to a column 0.9 × 60 cm and effluent fractions of 1.4 ml were collected at a rate of 4.2 ml/hr. ●——●, absorbance at 280 mμ; ○ - - - ○, enzyme activity.

with buffer (0.05 M Tris-HCl, 0.005 M $CaCl_2$, pH 7.5). After incubation at 37°C for 1 hr and then 4°C for 18 hr, precipitates were washed in 0.5 ml of cold 0.15 M NaCl. The precipitates were then dissolved in 0.5 N NaOH and their protein content determined. 0.1 ml portions of the supernatants were assayed for residual collagenase activity with ^{14}C-labeled fibrils as described below.

Preparation of tissue extracts. Approximately 10–20 g of rheumatoid synovial tissue obtained at synovectomy was trimmed of fat, minced finely with scissors, and homogenized in an all glass homogenizer at 0°C in 5.0 ml of 0.05 M Tris-HCl (pH 7.5) with 0.005 M $CaCl_2$. The homogenates were centrifuged at 10,000 *g* for 15 min at 4°C and the supernatants examined for enzymatic and immunologic evidence of collagenase.

Preparation of collagen substrate. Neutral salt extracted, ^{14}C-labeled collagen and acid-extracted unlabeled guinea pig skin collagen were purified according to the method of Gross (18, 19).

Assays. Collagenase activity was measured by the release of soluble glycine-^{14}C–containing peptides from native, reconstituted guinea pig skin collagen fibrils at 37°C (20). A typical reaction mixture consisted of 50 μl of 0.4% ^{14}C-labeled collagen, containing approximately 4000 cpm, 50 μl of 0.05 M Tris-HCl (pH 7.5), containing 0.005 M $CaCl_2$, and 100 μl of enzyme solution. Collagen purity was monitored by the addition of trypsin in a final concentration of 0.01% to an equal amount of ^{14}C-labeled collagen for the same time period. In every case, this trypsin blank represented less than 10% of the total counts. Noncollagenolytic protease activity was determined at pH 7.5 using casein as a substrate (21). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (22). Disc electrophoresis of the products of the reaction of collagenase and collagen at 28°C was performed according to the method of Nagai, Gross, and Piez (23).

Inhibition of synovial collagenase, by human whole serum and by the alpha globulins, was determined by the radioactive fibril assay after preincubation of whole serum or serum components at 37°C for 30 min. Alpha globulin was obtained as commercially prepared Cohn fraction IV (Hyland Laboratories). Partially purified α_2-macroglobulin and α_1-antitrypsin were prepared as previously described (10).

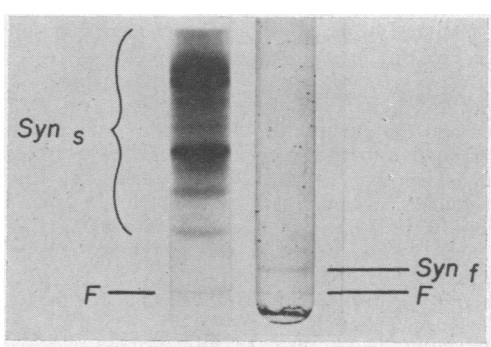

FIGURE 2 Polyacrylamide gel electrophoresis of synovial collagenase after chromatography on Sephadex G-75. The samples contained 75 μg of protein. Electrophoresis was carried out in 12.5% acrylamide at a constant current of 5 ma per tube. The gel on the left shows the heterogeneous slow-moving enzyme (Syn_s). On the right is the purified fast-moving enzyme (Syn_f). F, buffer front.

RESULTS

Purification and properties of synovial collagenase. Gel filtration on Sephadex G-75 of synovial collagenase, partially purified by passage through Sephadex G-150, results in the appearance of two distinct peaks of collagenase activity (Fig. 1). Peak I emerges with the void volume, whereas peak II is retarded by the G-75. On polyacrylamide gel electrophoresis, peak I consists of a slower moving heterogeneous group of proteins (Fig. 2) while peak II consists of a homogeneous, electrophoretically fast-moving protein, referred to as (Syn_f),[2] (Fig. 2). Although fast-moving synovial collagenase cannot be demonstrated in culture media of all patients examined, the slower moving component is always present.

The absence of fast-moving synovial collagenase from some synovial culture media prompted further purification of the slow-moving material by affinity chromatography. Fig. 3 demonstrates the appearance on a polyacrylamide gel of purified slow-moving rheumatoid synovial collagenase (Syn_s) eluted from collagen-Sepharose. This electrophoretically homogeneous protein was used as the immunogen in the preparation of anti-synovial collagenase (Syn_s) antiserum. No caseinolytic activity is present in the purified collagenase. In this highly purified state the enzyme is relatively unstable and loses approximately 75% of its activity after storage in solution for 2 wk at − 20°C.

Immunologic properties. On immunodiffusion, anti-Syn_s gamma globulin demonstrates a single precipitin band when reacted either with purified synovial collagenase (Syn_s), the immunogen, or with crude synovial

[2] *Abbreviations used in this paper:* aH_f, anti-human skin collagenase; anti-Syn_s, anti–slow-moving collagenase; Syn_f, fast-moving collagenase; Syn_s, slow-moving collagenase.

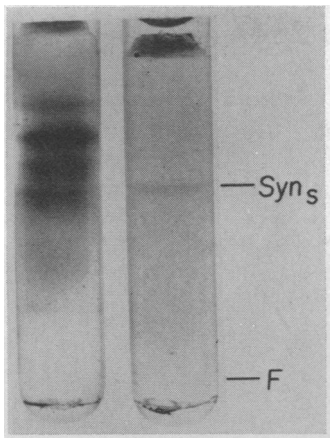

FIGURE 3 Polyacrylamide gel electrophoresis of slow-moving synovial collagenase purified on collagen-Sepharose. Samples contained 60–80 μg of protein. 12.5% acrylamide was used at 5 ma per tube. The gel on the left shows the pattern of unbound material from the first peak. On the right is the purified slow-moving enzyme (Syn$_s$) eluted from collagen-Sepharose with buffered 1.0 M NaCl. F, buffer front.

collagenase obtained from culture medium (Fig. 4A). When synovial collagenase (Syn$_s$) and human skin collagenase are reacted in a double diffusion experiment with anti-Syn$_s$ gamma globulin or antiserum to human skin collagenase (aH$_f$), a reaction of complete identity occurs (Fig. 4B). Similarly, fast moving (Syn$_f$) collagenase as well as the slow-moving enzyme (Syn$_s$), react with the anti-human skin collagenase (aH$_f$) with complete immunologic identity (Fig. 4C).

In order to determine the specificity of anti-synovial collagenase (Syn$_s$) antiserum for the enzyme, a quantitative precipitin reaction was performed in which enzyme activity was evaluated in the supernatants. In these studies the gamma globulin fraction was used so that possible antiprotease activity of other serum fractions, such as α_2-macroglobulin and α_1-antitrypsin, would be avoided. Fig. 5 demonstrates the ability of monospecific anti-Syn$_s$ gamma globulin to remove collagenase activity completely by precipitation. The points of minimum enzyme activity in the supernatant and maximum protein in the precipitate coincide, providing evidence that anti-Syn$_s$ antibody is specifically precipitating collagenase.

The inhibition of synovial collagenase by anti-Syn$_s$ antiserum was also confirmed by adding increasing amounts of gamma globulin to a constant amount of enzyme. After precipitation, collagenase activity in the supernatant decreases with increasing antibody concentration to a constant value of about 10% of the original activity. At this point, approximately 5 mg of anti-Syn$_s$ gamma globulin produces maximum inhibition of 0.18 mg of collagenase. A control preparation of an equal amount of nonimmune rabbit gamma globulin inhibits synovial collagenase only 3%.

Inhibition by serum components. Since human whole serum has been shown to be an effective inhibitor of synovial collagenase (2) and serum alpha globulin is a known inhibitor of both human skin and amphibian collagenases (10), the ability of the alpha globulin components of serum to inhibit synovial collagenase was investigated. Table I shows that enriched fractions of

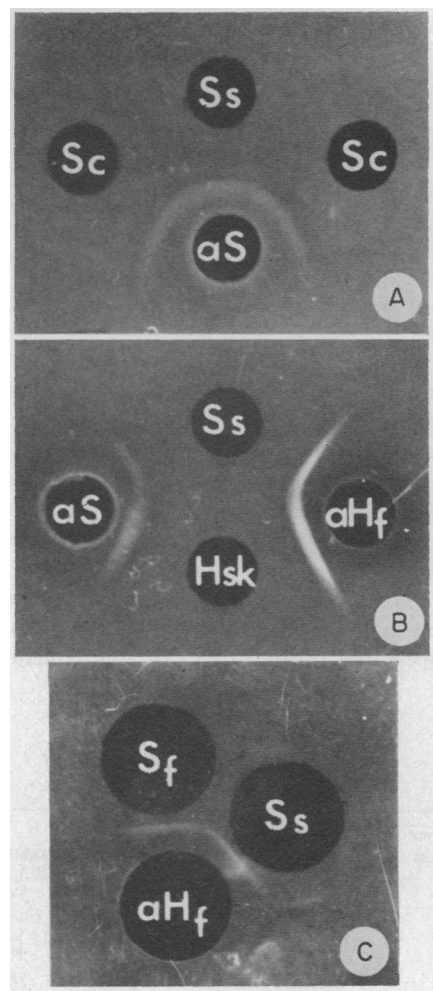

FIGURE 4 Gel diffusion analysis of antisera against rheumatoid synovial and human skin collagenases. (A) Sc, crude synovial collagenase from tissue culture; Ss, collagen-Sepharose purified slow-moving synovial collagenase; aS, anti–slow-moving synovial collagenase antiserum. (B) S$_s$, slow-moving synovial collagenase; aS, anti–slow-moving synovial collagenase antibody; Hsk, human skin collagenase; aH$_f$, antiserum to fast-moving human skin collagenase. (C) S$_s$, slow-moving synovial collagenase; S$_f$, fast-moving synovial collagenase; aH$_f$, antiserum to fast-moving human skin collagenase.

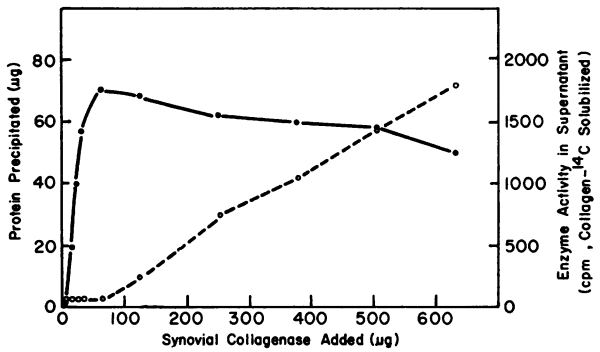

FIGURE 5 Quantitative precipitin reaction between slow-moving synovial collagenase and anti-slow-moving synovial collagenase gamma globulin. Increasing amounts of the enzyme were added to 0.1 ml of gamma globulin at a concentration of 25.0 mg protein/ml. After incubation, the protein content of precipitates was determined and collagenase activity in the supernatants measured by incubation of 0.1 ml portions on ^{14}C-labeled collagen fibrils for 2 hr at 37°C. A control reaction containing 126 µg of synovial collagenase and 0.1 ml of nonimmune rabbit gamma globulin (25.0 mg/ml) released 590 cpm. (See text for details.) ●——●, protein in precipitate; ○- - -○, collagenase activity in supernatant.

both α_2-macroglobulin and α_1-antitrypsin are, indeed, effective inhibitors of crude synovial collagenase. Studies are currently in progress directed at preparing highly purified preparations of both α_2-macroglobulin and α_1-antitrypsin to determine the stoichiometry of inhibition of purified synovial collagenase by these antiproteases.

Studies of synovial culture medium. Examination of culture medium for collagenase activity shows a delay in the appearance of the enzyme of approximately 24–48 hr, which is similar to that described by Evanson et al. (2). Low levels of enzyme activity can occasionally be detected in medium from the 1st or 2nd day of tissue culture. Daily examination of culture medium reveals that collagenase, as measured by its immunoreactivity, is present in the 24 and 48 hr medium as well as in enzymatically active media from days 3–10 (Fig. 6A).

TABLE I
Inhibition of Synovial Collagenase by Serum Alpha Globulins

Enzyme protein	Serum component	Protein	Inhibition
µg		µg	%
96	Alpha$_2$-M	235	73.4
96	Alpha$_2$-M	940	90.0
96	Alpha$_1$-at	74	86.4
96	Alpha$_1$-at	148	97.0

Reaction mixtures consisted of 50 µl of 0.4% glycine-^{14}C-labeled collagen as a substrate gel, 96 µg of synovial collagenase, and variable amounts of each of the alpha globulin components in a total volume of 150 µl. Incubation was carried out for 6 hr at 37°C in a shaking water bath. Alpha$_2$-M and Alpha$_1$-at refer to alpha$_2$-macroglobulin and alpha$_1$-antitrypsin.

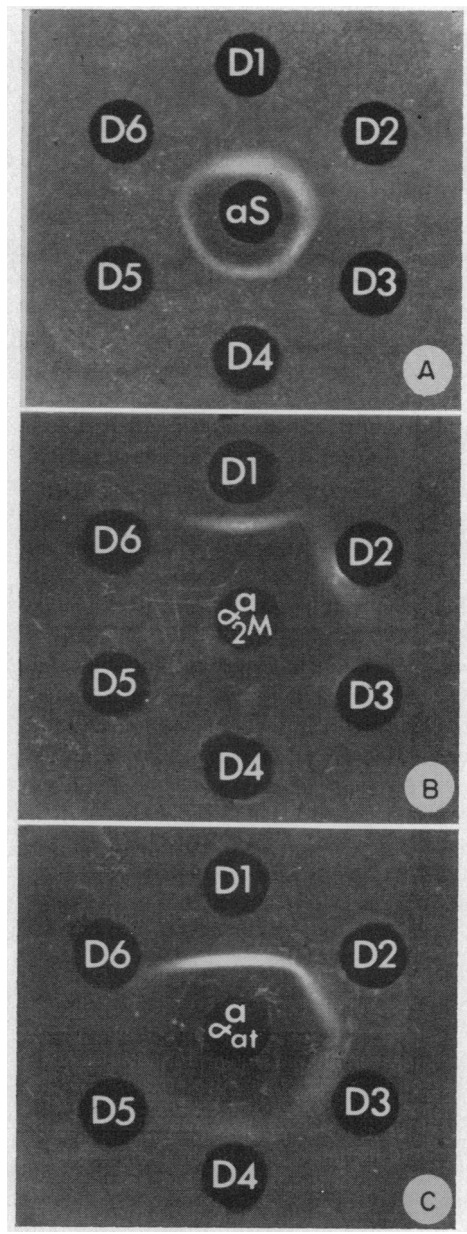

FIGURE 6 Immunodiffusion of synovial collagenase culture medium with antisera to synovial collagenase and to serum alpha globulins. D 1, D 2, D 3, D 4, D 5, D 6, medium from sequential days of culture. (A) aS, anti-slow-moving synovial collagenase antiserum. (B) aα_2M, anti-human α_2-macroglobulin. (C) a$\alpha_1$at, anti-human α_1-antitrypsin.

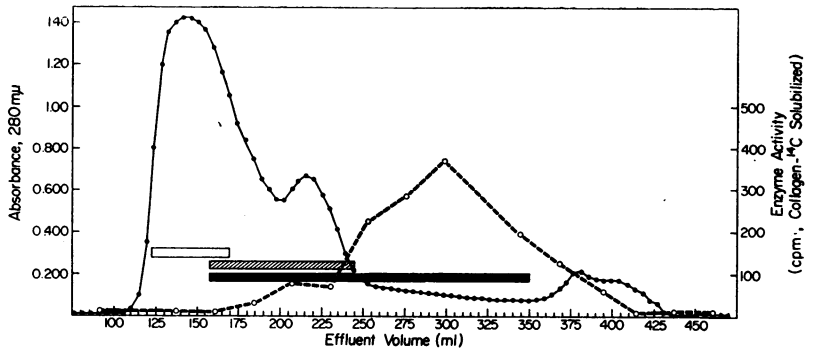

FIGURE 7 Gel filtration of day 1 culture medium on Sephadex G-150. A sample containing 135 mg of protein was applied to a column 2.5 × 100 cm at 4°C, and effluent fractions of 4.7 ml were collected at a rate of 14 ml/hr. ●——●, absorbance at 280 mμ; ○ - - - ○, enzyme activity; □, α_2-macroglobulin; ▒, α_1-antitrypsin; ■, immunoreactive synovial collagenase.

The protease inhibitors, α_2-macroglobulin, and α_1-antitrypsin, can be demonstrated primarily during the first 2 days of culture, a time when enzyme activity is either very low or undetectable (Fig. 6B, C).

When culture medium from the first 24 hr, which contains immunoreactive collagenase but little or no enzyme activity is subjected to gel filtration on Sephadex G-150, enzymatically active collagenase (Fig. 7) can be separated from the antiproteases, α_2-macroglobulin, and α_1-antitrypsin. Fig. 7 also shows that immunoreactive collagenase and low levels of enzyme activity are present in areas that overlap with the antiproteases, probably preventing recovery of all the enzymatic activity present in the preparation.

Synovial extracts. Although rheumatoid synovial extracts fail to demonstrate collagenase activity, the enzyme can be detected immunologically in these crude tissue extracts. Fig. 8 shows that the homogenate contains material which reacts with the monospecific antiserum to synovial collagenase (Syn$_s$). Furthermore, the crude synovial extracts give a precipitin pattern of identity when reacted in immunodiffusion with the purified synovial collagenase (Syn$_s$) obtained from tissue culture.

In preliminary experiments, using a synovial extract from one patient with rheumatoid arthritis, it has been possible to detect enzymatically active collagenase by gel filtration of the homogenate on Sephadex G-150 in a fashion similar to that described for extracts of human skin (9). Recovery of active synovial collagenase appears to depend on the chromatographic separation of collagenase from the alpha globulins. When this enzymatically active fraction is reacted with collagen in solution at 28°C and the reaction products are examined by disc gel electrophoresis, faster moving components can be seen when the specific viscosity has decreased by approximately 20% (Fig. 9). This pattern is essentially identical with that previously described for synovial collagenase from tissue culture (1).

DISCUSSION

The demonstration that collagenase is present in vivo in human skin (9) strengthens the concept that col-

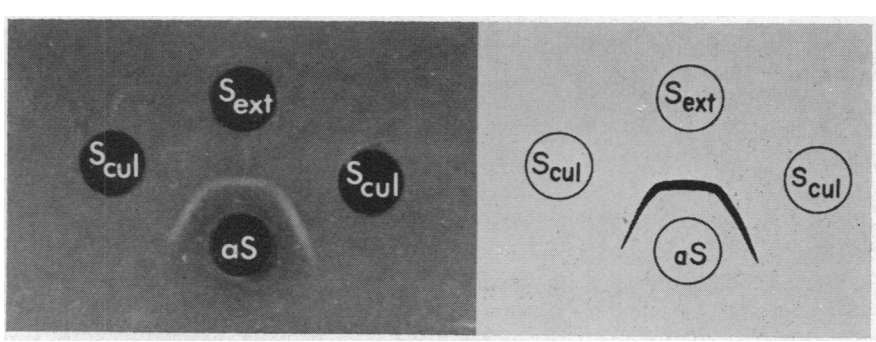

FIGURE 8 Agar gel diffusion of a crude tissue extract of rheumatoid synovium and synovial collagenase obtained from tissue culture against anti-slow-moving synovial collagenase antiserum. Scul, synovial collagenase from culture medium; Sext, rheumatoid synovial extract; aS, anti-synovial collagenase antibody.

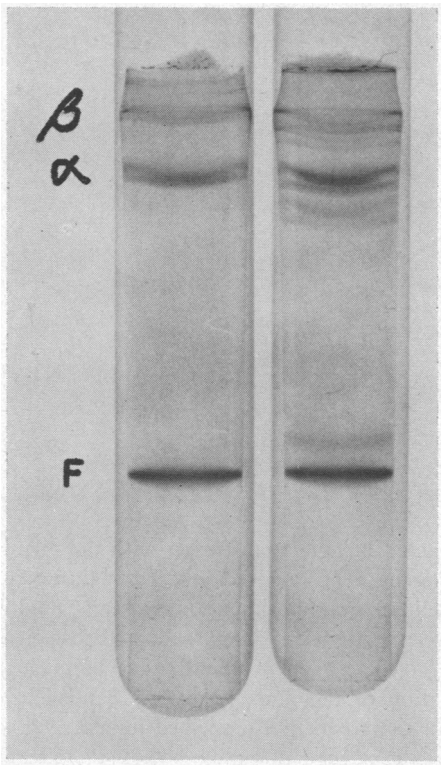

FIGURE 9 Acrylamide gel electrophoresis of extracted rheumatoid synovial collagenase–collagen reaction mixture after thermal denaturation. The collagenase was prepared by passage of a crude extract of rheumatoid synovium through Sephadex G-150. (See text.) On the left is the 0 time reaction mixture; on the right, after a 21% reduction in specific viscosity. α refers to the single polypeptide chain and β to the cross-linked dimers of the α-chains. F, buffer front. An identical pattern is obtained with collagenase from day 1 culture medium after gel filtration on Sephadex G-150.

lagenases are not only of physiologic significance in the normal processes of collagen remodeling but that they may also play a role in diseases associated with enhanced collagen degradation. In addition, a number of studies (9, 11)[3, 4] suggest that the in vitro system for collagenase production may provide an excellent experimental approach to the study of collagen remodeling.

In tissue cultures of rheumatoid synovial explants, examination of the medium reveals that collagenase is, indeed, present during the first 2 days of culture, but can only be detected immunologically at this time. On subsequent days (Fig. 6A), however, both immunologically reactive and enzymatically active collagenase is found in the culture medium. The serum protease inhibitors, α_2-macroglobulin and α_1-antitrypsin, are present primarily during the first 2 days of tissue culture, suggesting that the failure to detect collagenase activity during the first 24–48 hr of culture is due to the presence of these inhibitory alpha globulins, which are presumably present in the synovial tissue at the time it is placed in serum-free culture medium. After several changes of medium, the concentration of alpha globulin is apparently lowered sufficiently to allow enzyme activity to be detected.

When enzymatically inactive medium of rheumatoid synovial explants is taken from the 1st day of culture and subjected to gel filtration on Sephadex G-150, collagenase activity can be detected. This demonstration of collagenase activity appears to be closely related to the separation of the enzyme from α_2-macroglobulin and α_1-antitrypsin, indicating that the absence of enzymatic activity during the first 24–48 hr of tissue culture is related to the presence of these alpha globulins in the culture medium. The in vitro findings for rheumatoid synovial collagenase are similar to those reported for human skin collagenase (9).

In the present investigation, it has also been possible to demonstrate the presence of immunoreactive collagenase in extracts of rheumatoid synovial tissue which have no detectable collagenase activity either against collagen in solution or reconstituted native collagen fibrils. This was accomplished using a monospecific antiserum prepared against rheumatoid synovial collagenase purified by affinity chromatography from tissue culture medium containing synovial explants. The precipitin reaction on immunodiffusion, between purified synovial collagenase and immunoreactive material in rheumatoid synovial extracts, demonstrates the presence in the tissue of collagenase which is immunologically identical to collagenase from tissue culture. Furthermore, preliminary observations indicate that enzymatic activity can be recovered from rheumatoid synovial extracts by gel filtration on Sephadex G-150. The presence of both α_2-macroglobulin and α_1-antitrypsin in tissue extracts of rheumatoid synovium suggests that collagenase activity in such extracts may also be masked by the inhibitory action of these alpha globulins. The role of each of these alpha globulins as inhibitors of synovial collagenase can be determined when the stoichiometry of enzyme to inhibitor is assessed with highly purified preparations of both α_2-macroglobulin and α_1-antitrypsin.

Recent immunologic studies have shown that species specificity exists between human and animal collagenases (7). By utilizing antiserum to purified rheumatoid synovial collagenase, these findings have been extended to show that rheumatoid synovial and human skin collagenases share major antigenic determinants, indicating

[3] Jeffrey, J. J., R. J. Coffey, and A. Z. Eisen. Studies on collagenase in tissue culture I. Relationship of enzyme production to collagen metabolism. *Biochim. Biophys. Acta.* In press.

[4] Jeffrey, J. J., R. J. Coffey, and A. Z. Eisen. Studies on collagenase in tissue culture II. Effect of steroid hormones on enzyme production. *Biochim. Biophys. Acta.* In press.

that they are immunologically identical and that organ specificity for collagenases from these tissues is unlikely. Further comparative studies on these purified enzymes are currently in progress.

Of interest is the demonstration that two distinct species of rheumatoid synovial collagenases are present in the tissue culture medium. Although the fast-moving synovial collagenase (Syn_f) is variably present in culture medium of synovial explants, like its counterpart from human skin cultures (7), it can be obtained as an electrophoretically homogeneous protein by sequential gel filtration on Sephadex G-150 and G-75. The molecular weight of the fast-moving synovial collagenase (Syn_f) has not as yet been determined, but its gel filtration characteristics on Sephadex G-75 as well as its electrophoretic migration near the front on polyacrylamide gel electrophoresis, indicate that it has a significantly lower molecular weight than slow-moving synovial collagenase (Syn_s). Whether a monomer-dimer relationship exists between the fast- and slow-moving enzymes is unknown.

The relationship of the two synovial enzymes obtained from tissue culture (Syn_s and Syn_f) to the presence of the two species of collagenase identified by Harris et al. (4) in some rheumatoid synovial fluids is unclear. Synovial fluid collagenase "A" of Harris et al. is an enzyme of approximately 50,000 mol wt and is not inhibited by serum. Synovial fluid collagenase "B," an enzyme of approximately 25,000 mol wt, is inhibited by serum. The results of the present study show that human whole serum is capable of inhibiting both the purified fast-moving (Syn_f) and slow-moving (Syn_s) synovial collagenases (Table II). Some variability is seen in the quantitative inhibition of these synovial collagenases with the electrophoretically fast-moving collagenase being more readily inhibited than the slow-moving enzyme. Until additional information is available concerning the biochemical nature of the collagenases obtained from rheumatoid synovial tissue cultures and from rheumatoid synovial fluid, a precise comparison of these enzymes is not possible.

The availability of a monospecific antiserum to rheumatoid synovial collagenase that reacts with identity to enzyme present in the tissue suggests that an immunologic approach may yield valuable information regarding the in vivo role of collagenase in the pathogenesis of rheumatoid arthritis. Although Harris et al. (4) have demonstrated that in an individual patient with rheumatoid arthritis, collagenase activity cannot always be demonstrated in synovial fluid, our preliminary observations [5] indicate that immunoreactive collagenase is present in the joint fluid of patients with rheumatoid arthritis in the absence of demonstrable enzyme activity.

[5] Bauer, E. A., A. Z. Eisen, and J. J. Jeffrey. Unpublished observations.

TABLE II

Inhibition of Purified Synovial Collagenases by Human Serum

Enzyme	Enzyme protein	Serum protein	S/E*	Inhibition
	μg	μg		%
Syn_f	54	150	3	50.3
Syn_s	44	750	17	69.1
Syn_s	103	7500	73	77.3

Reaction mixtures consisted of 50 μl of 0.4% glycine-^{14}C-labeled collagen as a substrate gel and variable amounts of collagenase and serum protein in a total volume of 200 μl. Incubation was carried out for 6 hr at 37°C in a shaking water bath. Syn_f and Syn_s refer to purified preparations of fast- and slow-moving synovial collagenase.

* Ratio of serum protein/enzyme protein.

This inability to detect collagenase activity may, indeed, be related to the level of serum antiproteases in synovial fluid.

ACKNOWLEDGMENTS

Our appreciation goes to Miss Patricia McGowen for her excellent technical assistance, and to the members of the Department of Orthopedic Surgery, who provided rheumatoid synovium.

This work was supported by the United States Public Health Service Research Grants AM 12129 and AM 05611, from the National Institute of Arthritis and Metabolic Diseases.

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