Immunochemical Studies with a Specific Antiserum to Rabbit Fibroblast Collagenase

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1. Antisera were raised against the collagenase from rabbit synovial fibroblasts, and characterized by immunoprecipitation and immunoinhibition reactions. 2. Immunoglobulins from the antisera were potent inhibitors of the action of rabbit collagenase on both reconstituted collagen fibrils and collagen in solution. 3. The antibody-binding fragment, Fab', produced by digesting the IgG (immunoglobulin G) with pepsin, inhibited collagenase activity just as well as whole IgG. 4. A specific antiserum to the rabbit collagenase was raised by a multi-step procedure. An initial antiserum was made by injecting partially purified collagenase as a complex with sheep α_2 -macroglobulin into a sheep. The non-specific antiserum so obtained was used to produce precipitin lines with the purified enzyme, and these lines were used as antigen for the production of the specific antiserum. 5. An IgG preparation from the specific antiserum was a specific and potent inhibitor of the rabbit synovial fibroblast collagenase. Neutral metallo-proteinase activity secreted by the rabbit fibroblasts was not inhibited by the antibody to the rabbit collagenase. 6. Criteria for determination of the specificity of antisera are discussed.

A specific antiserum directed against a tissue proteinase is an important tool in attempting to elucidate the function of these enzymes in the degradation of connective tissue macromolecules. Extensive studies on collagenase have implicated this enzyme in the physiological and pathological breakdown of collagen (reviewed by Harris & Krane, 1974*a,b,c*). There are many reports in the literature on the purification of specific collagenases and on attempts to make antisera, but so far there have been no adequate reports on a specific anti-collagenase antiserum. The preceding paper (Werb & Reynolds, 1975) describes the purification and characterization of a specific collagenase from rabbit fibroblasts growing in culture, and in the present paper we describe the preparation of specific antisera to rabbit collagenase and detailed experiments on the immunoinhibition by these antisera.

Materials and Methods

Materials

Materials were as listed in the preceding paper (Werb & Reynolds, 1975), with the following additions. Antisera to sheep serum proteins and human α_2 -macroglobulin were prepared by Dakopatts, Copenhagen, Denmark and purchased from Mercia Diagnostics Ltd., Watford, Herts., U.K. DEAE-cellulose (Whatman DE-52) was obtained from Reeve Angel Scientific, London EC4U 6AY, U.K. Bovine achilles-tendon collagen and agarose were purchased

from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sheep antiserum to rabbit cathepsin D (D483/2) was the gift of Dr. J. T. Dingle, Strangeways Research Laboratory, Cambridge, U.K.

Methods

Preparation of collagenase. Rabbit synovial fibroblasts were obtained from normal rabbit synovium as described by Werb & Burleigh (1974). Collagenase activity in the conditioned serum-free culture medium of these cells was concentrated by using a Sartorius ultrafiltration membrane in a Chemlab ultrafiltration cell (Chemlab Instruments, London IG3 8BT, U.K.) and purified by gel filtration and ion-exchange chromatography (procedures I or II of Werb & Reynolds, 1975).

Collagenase assays. Assays of collagenase activity with reconstituted collagen fibrils, and with collagen in solution at 35°C, were made as described previously (Werb & Burleigh, 1974; Werb & Reynolds, 1975; McCroskery *et al.*, 1973). For immunoinhibition studies the enzyme sample was mixed immediately before assay with various concentrations of the test IgG* or Fab', then assayed in the usual way.

Neutral proteinase assays. Assays for neutral proteinase were made by using either azocasein (Werb

* Abbreviations: IgG, immunoglobulin G; F(ab')₂, bivalent antibody binding fragment produced by pepsin digestion of IgG; Fab', univalent antibody binding fragment produced by reduction of F(ab')₂.

et al., 1974) or gelatin (Werb & Burleigh, 1974). Assays were for 4 h at 37°C. For immunoinhibition studies the enzyme sample was mixed immediately before assay with various concentrations of IgG. All assays were made under conditions where the degradation of the substrate was linear with respect to enzyme concentrations and time.

Test of specificity of antisera. Antisera raised against rabbit fibroblast collagenase were tested for specificity by double immunodiffusion against normal rabbit serum, sheep serum, foetal calf serum, rabbit collagen, various preparations of crude and purified rabbit synovial cell collagenase, and pools of proteins separated from the collagenase during the purification procedure.

Preparation of a non-specific antiserum to rabbit collagenase. Rabbit collagenase was purified by Procedure I (QAE-Sephadex method) of Werb & Reynolds (1975). A sheep (X311) was injected intramuscularly three times at 2-week intervals with 95 µg of collagenase (1730 units/mg) emulsified with Freund's complete adjuvant, and exsanguinated 60 days after the initial injection. [One unit of collagenase activity is defined as the solubilization of 1 µg (3.3 pmol) of reconstituted rabbit collagen fibrils/min at 37°C.] The antisera are designated by the number of the animal, followed by a stroke and the number of the bleed from which the serum was derived (e.g. X311/4).

Preparation of a specific antiserum to rabbit collagenase. The specific antiserum to rabbit fibroblast collagenase was raised in sheep by a three-part procedure. First the enzyme was further purified by re-isolation of it as its α_2 -macroglobulin complex (Werb *et al.*, 1974), and this was used as the immunogen. The antibodies to collagenase were isolated from this antiserum by immunoprecipitation with purified collagenase in gels, with the immunoprecipitates being used as antigen in a second animal. This precipitin procedure was repeated again and used as antigen in a third animal, with a resulting specific antiserum (see Table 1).

Preparation of collagenase- α_2 -macroglobulin complexes as antigen. A sheep α_2 -macroglobulin fraction was prepared by gel filtration of normal sheep serum obtained from a preimmunization bleed of the same sheep (Z10) to be used to prepare the antiserum. Serum (8 ml) was applied to a column (2.5 cm × 45 cm) of Sepharose 6B equilibrated with 100 mM-Tris-HCl buffer, pH 7.3. Fractions (6 ml) were collected at 85 ml/h and the sheep α_2 -macroglobulin was identified by double immunodiffusion against rabbit anti-(human α_2 -macroglobulin); this antiserum gave cross-reactions with α_2 -macroglobulin from human, sheep and bovine sera. The sheep α_2 -macroglobulin was eluted just after the void volume and fractions 17–20 were pooled. After concentration by ultrafiltration the α_2 -macroglobulin

preparation (5 ml; approx. 4 × concentrated) was mixed with 2.4 mg of partially purified collagenase (stage 2, approx. 130 units/mg) in 5 ml of 50 mM-Tris-HCl buffer, pH 7.6, containing 5 mM-CaCl₂. The mixture was incubated at room temperature (22°C) for 100 min to allow the enzyme to react with the α_2 -macroglobulin, then rechromatographed on the Sepharose 6B. Fractions containing α_2 -macroglobulin (fractions 16–25) were pooled and concentrated to 12 ml by ultrafiltration. No collagenase activity was detected in the α_2 -macroglobulin complex or at the elution volume for collagenase (fractions 33–38 on a separate chromatogram). The sheep (Z10) from which the α_2 -macroglobulin was derived was injected intramuscularly on day 0 with 4 ml of the collagenase- α_2 -macroglobulin complex emulsified with 4 ml of Freund's complete adjuvant. The procedure was repeated after 15 and 30 days and the animal was then exsanguinated 15 days later. The antiserum so obtained gave a single precipitin line on double immunodiffusion against the collagenase preparation used initially to make the α_2 -macroglobulin complex. However, one, and possibly two, additional precipitin lines could be discerned by using a highly concentrated partially purified collagenase preparation (0.59 mg/ml; approx. 300 units/mg). Immunoglobulin isolated from this antiserum inhibited the activity of the rabbit fibroblast collagenase in the assay with radioactive fibrils.

Purification of specific antibodies to the collagenase as precipitin lines. Precipitin lines were used to purify both collagenase and its specific antibodies (Shivers & James, 1967; Weston, 1969; Lachman, 1970; Dean, 1974). Precipitin lines were prepared by reacting purified collagenase [stage II-3 (Werb & Reynolds, 1975) 59 units/ml] with the antiserum raised to the α_2 -macroglobulin-collagenase complex (Z10) in alternating troughs (8 mm, centre to centre) on 1% (w/v) agarose plates buffered with 50 mM-Tris-HCl buffer, pH 7.6, containing 100 mM-NaCl, 5 mM-CaCl₂ and 0.01% NaN₃. These conditions of antibody/antigen ratio produced precipitates at equivalence for the collagenase-anticollagenase, and soluble immune complexes or well-separated precipitin lines for the contaminants. Plates were washed for 2 days in the same buffer, then the immunoprecipitate lines were cut out carefully and washed for 2 days with five buffer changes. For each injection ten such lines were emulsified with 4 ml of Freund's complete adjuvant by 2 × 5 s bursts of a small Ultra-Turrax homogenizer, and placed in two intramuscular sites of a sheep (A3) on day 0, and again on day 15. A trial bleed taken on day 16 gave only a single line on double immunodiffusion. However, at exsanguination on day 33 the antiserum was no longer specific, and hence a second series of precipitin lines were prepared by reacting the specific antiserum from the early bleed of sheep A3

with rabbit fibroblast collagenase (stage II-4; 44 µg/ml). Eight precipitin lines were emulsified with 4 ml of Freund's complete adjuvant and injected into a sheep (V436) on day 0. The injection was repeated on day 15 and a large bleed was taken on day 40. The sheep was given a third injection of precipitin lines on day 70 and the animal bled out on day 120. The antisera derived from various bleeds from this sheep were all specific as judged by critical immunodiffusion tests, and IgG from these antisera inhibited collagenase activity (see the Results section).

Preparation of immunoglobulins from sheep antisera. $(\text{NH}_4)_2\text{SO}_4$ precipitation. Crude IgG preparations were made by adding 1 vol. of 4 M- $(\text{NH}_4)_2\text{SO}_4$, pH 7.0, to 2 vol. of sheep serum. The precipitate was redissolved in water to the original serum volume, and then the precipitation step repeated twice. The final precipitate was dissolved in a minimum volume of 50 mM-Tris-HCl buffer, pH 7.6, and dialysed against this buffer.

Purified IgG was prepared by chromatography of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated globulins on DE-52 cellulose, essentially as described by Aalund *et al.* (1965). IgG was identified and checked for purity by immunoelectrophoresis against rabbit antiserum to sheep serum proteins. Samples for use in immunoinhibition experiments were dialysed against 50 mM-Tris-HCl buffer, pH 7.6.

Fab' fragments of immune and non-immune sheep IgG were prepared by digesting with pepsin the IgG prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Nisonoff *et al.*, 1960). After digestion (24 h at 37°C) the pepsin was inactivated by adjusting the pH of the reaction mixture to pH 8.6, and the required product was purified from undigested IgG and other digestion products by chromatography on Sephadex G-100, after reduction to fragment Fab' with 1 mM-dithiothreitol. Since fragment Fab' was not alkylated as a routine, some $\text{F}(\text{ab}')_2$ was formed after the removal of dithiothreitol. In experiments with fragment Fab' the $\text{F}(\text{ab}')_2$ -fragment content was less than 25% of the total protein, as judged by gel filtration on Sephadex G-100. The purity of the Fab' fragment was checked by immunoelectrophoresis against rabbit antiserum against sheep serum proteins. Before using the Fab' fragments in immunoinhibition experiments the dithiothreitol was removed by dialysing against 50 mM-Tris-HCl buffer, pH 7.6.

Other immunological techniques. Immunoglobulin concentrations were measured by E_{280} , calculating protein concentration from $E_{280}^{1\%} = 14$ (Kirschenbaum, 1970). Concentrations were checked by radial immunodiffusion in an agarose gel containing 5% (v/v) of an antiserum against sheep immunoglobulins, by the method of Mancini *et al.* (1965).

Double immunodiffusion (Ouchterlony, 1967; Clausen, 1969) was made with 1% (w/v) agarose in 50 mM-Tris-HCl buffer, pH 7.6, containing 0.2 M-

NaCl, and 5 mM- CaCl_2 . Plates were stained for protein with Coomassie Brilliant Blue R250 after washing for 48 h in 1% (w/v) NaCl containing 1% (v/v) butan-1-ol, and drying as described by Dean (1974).

For immunoelectrophoresis at pH 8.5, 1% (w/v) agarose plates were prepared as described previously (Werb & Reynolds, 1975).

Quantitative immunoprecipitation reactions were made in microcentrifuge tubes. Various amounts of immune and non-immune IgG were added to tubes containing collagenase. Mixtures were incubated for 4 h at 25°C followed by 16 h at 4°C. After centrifuging at 10000g for 10 min the free enzyme activities in the supernatant solution were determined in the assay with radioactive collagen fibrils. Total collagenase activity in the supernatants and in the precipitates was determined by assaying for the release of hydroxyproline-containing peptides from insoluble bovine tendon collagen at pH 7.6 as described previously (Werb & Burleigh, 1974; Burleigh *et al.*, 1974), except that assays were made in the presence of 5 M-urea to dissociate the enzyme-antibody complex. The collagenase was stable in the presence of 5 M-urea if other proteins were also present. Products of the reaction were denatured by heating in urea and analysed by polyacrylamide-gel electrophoresis at pH 3.5.

Measurements of protein in immunoprecipitates were made after dissolving the precipitates in 1 M-NaOH (Lowry *et al.*, 1951), with crystalline bovine serum albumin (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) as standard.

Immunoelectrophoretic assays ('rockets') of rabbit collagenase using sheep anti-(rabbit collagenase) antiserum were made essentially by the method of Laurell (1972; also see Axelsson *et al.*, 1973). Immune serum (5%, v/v) was added to 1% (w/v) agarose in a buffer consisting of 75 mM-Tris, 25 mM-diethylbarbituric acid and 5 mM- CaCl_2 (pH 8.6). Samples were placed in 5 µl (2 mm diam.) wells and samples electrophoresed towards the anode at 10 V/cm for 16 h.

All solutions contained either 0.02% sodium azide or 1% buton-1-ol as preservative.

Results

Preparation of a specific antiserum to rabbit synovial-cell collagenase

General considerations. Attempts to raise a specific antiserum directly by the injection of purified collagenase into sheep were unsuccessful. Tests for immunological purity are quite different from biochemical tests of enzyme purity since trace components, especially of serum proteins, can be highly immunogenic. The procedure adopted combined the preparation of an antiserum to collagenase-sheep

Table 1. *Summary of the procedure for preparation of a specific antiserum to rabbit fibroblast collagenase*

Experimental details are given in the text and quantitative results are presented in Plate 1 and Table 2.

Stage	Antigen	Antiserum prepared	Specificity
1	α_2 -macroglobulin-collagenase complexes	Z10/	Nonspecific
2	Precipitin lines prepared from Z10/4 and purified collagenase	A3/	Specific and non-specific bleeds
3	Precipitin lines prepared from A3/2 and purified collagenase	V436/	Specific

α_2 -macroglobulin complexes, with the purification of specific antibody-enzyme immunoprecipitates for use as immunogens (Table 1, and see the Materials and Methods section). Since there is no specific staining procedure for collagenase, the criterion for an antiserum to collagenase was the production of IgG inhibiting the specific enzymic activity of collagenase.

Antiserum raised against sheep α_2 -macroglobulin-rabbit collagenase complexes. The method for preparation of the first antiserum exploited a further purification of the collagenase preparation, based on the studies showing that only active endopeptidases are able to bind to α_2 -macroglobulin (Barrett & Starkey, 1973; Werb *et al.*, 1974). An α_2 -macroglobulin-enriched fraction was isolated from sheep serum and this was reacted with partially purified collagenase (see the Materials and Methods section for details), and the α_2 -macroglobulin fraction with bound collagenase re-isolated. As shown in Plate 1(a) an antiserum to human α_2 -macroglobulin cross-reacted with sheep α_2 -macroglobulin and this was used to identify the sheep α_2 -macroglobulin-collagenase complexes. Active collagenase was recovered after dialysis of the α_2 -macroglobulin-collagenase complex against 3M-NaSCN as described previously (Abe & Nagai, 1972; Werb *et al.*, 1974). When used as antigen the α_2 -macroglobulin complexes gave rise to an inhibitory and precipitating antiserum, but several lines could be discerned in immunodiffusion under certain conditions. As shown in Plate 1(b) the antiserum Z10/4 gave precipitin lines with purified collagenase but not with sheep α_2 -macroglobulin-collagenase complexes used as immunogen. It has also been observed that α_2 -macroglobulin-elastase complexes do not cross-react with anti-elastase antisera (Katayama & Fujita, 1974).

The procedure of preparing α_2 -macroglobulin-collagenase complexes as antigen was used successfully on a second occasion to raise an inhibitory antiserum to rabbit collagenase.

Antisera raised by using immune precipitin lines with purified collagenase. The preparation of precipitin lines between purified collagenase and the non-specific antiserum (Z10/4) under equivalence conditions giving only the specific precipitate for the enzyme is illustrated in Plate 1(c). Assuming that 90% of the collagenase used was bound in the precipitin lines at equivalence, then the visible precipitates in Plate 1(c) contained only about 2-4 μ g of enzyme protein with the majority of the protein seen being IgG.

The procedure used in the preparation of specific antisera to collagenase (A3, bleeds 1 and 2, and V436, all bleeds) is shown in Table 1. Plate 2(c) shows the precipitating capacity of the various specific antisera from sheep V436 as shown by double immunodiffusion against crude collagenase. These data demonstrate that non-specific antisera precipitate collagenase, but only the specific antisera give a single line with crude collagenase. An immunoplate summarizing the 'multigeneration' preparation of a specific antiserum to rabbit fibroblast collagenase is shown in Plate 2(d).

Criteria for specificity of the sheep-anti(rabbit collagenase) antisera. The antisera judged as specific (A3/1, 2; V436, all bleeds) gave one line only in double immunodiffusion against purified and crude rabbit fibroblast collagenase over tenfold ranges of concentrations of either enzyme or antisera. All plates were examined by dark-ground illumination, and again after staining for protein with Coomassie Brilliant Blue R250 (Werb & Reynolds, 1975). No lines were visible when the antisera were tested with rabbit serum, sheep serum, foetal calf serum, rabbit skin collagen and various contaminating protein pools obtained during the purification of the collagenase (Werb & Reynolds, 1975).

Inhibitory capacity of various sheep antisera to rabbit fibroblast collagenase. The inhibition of collagenase by IgG from the various antisera raised against this enzyme, is shown in Table 2. The ability of the IgG to produce 50% inhibition of the collagenase was unrelated to whether the antiserum was either specific or non-specific.

Inhibitory capacity of the specific antiserum to rabbit fibroblast collagenase for rabbit neutral proteinases. The specificity of the immunoinhibition of the antiserum directed against rabbit fibroblast collagenase was examined next. IgG prepared from the specific sheep anti-(rabbit collagenase) antiserum, V436/4, was tested for its capacity to inhibit rabbit fibroblast collagenase and the neutral metalloproteinase(s) separated from the culture fluid of rabbit fibroblasts. As Table 3 shows, only the collagenase activity was inhibited by the immune IgG, whereas all the enzymic activities were inhibited by EDTA (trisodium salt) and 1,10-phenanthroline.

EXPLANATION OF PLATE I

Immunodiffusion plates

(a) Cross-reactivity of human and sheep α_2 -macroglobulin. As, Rabbit anti-(human α_2 -macroglobulin); well 1, human α_2 -macroglobulin; well 2, sheep α_2 -macroglobulin; well 3, rabbit collagenase; well 4, sheep α_2 -macroglobulin-rabbit collagenase complexes. (b) Immunoreactivity of α_2 -macroglobulin-collagenase complexes. As, Specific sheep anti-(rabbit collagenase), A3/2; well 1, impure rabbit fibroblast collagenase; well 2, sheep α_2 -macroglobulin-collagenase complexes; well 3, sheep α_2 -macroglobulin-collagenase complexes treated with 3M-NaSCN. (c) Straight precipitin lines for injection, formed between troughs filled alternatively with either non-specific antiserum to rabbit collagenase, Z10/4, or purified rabbit fibroblast collagenase (stage II-4).

EXPLANATION OF PLATE 2

Immunodiffusion plates

(a) Stages in the preparation of a specific antiserum to rabbit fibroblast collagenase. Enz, Partially purified rabbit fibroblast collagenase; well 1, non-specific antiserum raised against collagenase-sheep α_2 -macroglobulin complexes, Z10/4; well 2, specific antiserum, A3/2, raised against immunoprecipitin lines prepared from collagenase and Z10/4; well 3, specific antiserum V436/4, raised against immunoprecipitin lines prepared from collagenase and A3/2; well 4, non-specific antiserum to rabbit collagenase, X311/4. Wells contain collagenase at various stages of purification (see Werb & Reynolds, 1975); well 1, unconcentrated culture medium (stage 0); well 2, concentrated culture medium (stage 1); well 3, Sephadex G-100 purified collagenase (stage 2); well 4, enzyme as in well 6, but concentrated by Biorex 70 (stage II-4); well 5, neutral proteinase fraction from Biorex 70 chromatography; well 6, purified collagenase from Biorex 70 chromatography (stage II-3).

(b) Enz, Concentrated culture medium; wells contain various antisera to rabbit collagenase; well 1, non-specific antiserum X311/4; well 2, non-specific antiserum A3/4; well 3, specific antiserum A3/2; well 4, specific antiserum V436/4. Wells contain collagenase at various stages of purification (see Werb & Reynolds, 1975); well 1, unconcentrated culture medium (stage 0); well 2, concentrated culture medium (stage 1); well 3, Sephadex G-100 purified collagenase (stage 2); well 4, enzyme as in well 6, but concentrated by Biorex 70 (stage II-4); well 5, neutral proteinase fraction from Biorex 70 chromatography; well 6, purified collagenase from Biorex 70 chromatography (stage II-3).

(c) Enz, Partially purified rabbit collagenase (stage 2); wells contain various bleeds of sheep V436, injected with precipitin lines containing collagenase and its specific antibody (numbers correspond to the number of bleed). All bleeds can be seen to be specific.

(d) Enz, Concentrated culture medium; wells contain various antisera to rabbit collagenase; well 1, non-specific antiserum X311/4; well 2, non-specific antiserum A3/4; well 3, specific antiserum A3/2; well 4, specific antiserum V436/4.

(e) Immuno-electrophoretic assays of rabbit collagenase using agarose gel containing 5% (v/v) of specific antiserum to rabbit collagenase, V436/7. Wells contained the amounts of concentrated culture medium (Enz) shown (see the Materials and Methods section).

(f) Immuno-electrophoretic assay of rabbit collagenase using a non-specific antiserum (X311/4) to rabbit collagenase. Conditions similar to (e) but with 5 μ l of concentrated culture medium. The lack of specificity is clearly seen, as compared with (e).

Table 2. *Inhibitory capacity of various sheep antisera to rabbit fibroblast collagenase*

IgG was prepared from the antisera by $(\text{NH}_4)_2\text{SO}_4$ precipitation and used in immunoinhibition studies as described in the Materials and Methods section. Equivalent antiserum concentrations in the assay were calculated in terms of μl in a $300\mu\text{l}$ assay with radioactive reconstituted collagen fibrils. Collagenase (0.05 units) was added to each assay tube and incubations were for 18 h. s, Specific; n.s., non-specific.

Antiserum	Specificity of antiserum	Antiserum concentration giving 50% inhibition (μl per tube)
X311/4	n.s.	0.25
Z10/3	n.s.	8.0
Z10/4	n.s.	2.0
A3/2	s	2.0
A3/4	n.s.	0.75
V436/2	s	1.5
V436/4	s	0.5
V436/7	s	1.0
Non-immune sheep serum	—	>300

Table 3. *Comparison of the inhibition of rabbit fibroblast metallo-proteinases by sheep antiserum to rabbit fibroblast collagenase and chelating agents*

IgG was prepared from the specific antiserum to rabbit fibroblast collagenase (V436/4) and used for immunoinhibition studies as described in the Materials and Methods section. Collagenase activity was assayed with reconstituted collagen fibrils; neutral proteinase activity was measured by using either $[^{14}\text{C}]$ glycine-labelled gelatin or azocasein. For tests of inhibition by chelators, EDTA (trisodium salt; 20 mM in the presence of 10 mM CaCl_2) or 1,10-phenanthroline (1 mM) was mixed with the enzyme immediately before assay. The assays were made with collagenase and proteinase preparations separated on Biorex 70 (Werb & Reynolds, 1975).

Compound	Enzymic activity (% of control)		
	Collagenase	Neutral proteinase	
		Gelatin	Azocasein
IgG (normal sheep)			
0.85 mg/ml	100	102	106
0.08 mg/ml	96	105	99
IgG (V436/4)			
0.83 mg/ml	6	96	104
0.08 mg/ml	59	102	94
20 mM-EDTA (trisodium salt)	2	0	4
1 mM-1,10-Phenanthroline	5	2	11

In another experiment the differential inhibition of collagenase and gelatinase activity present in crude culture medium was demonstrated. Portions of

conditioned medium were assayed for enzymic activity on either reconstituted collagen fibrils or gelatin in the presence of $100\mu\text{g}$ of IgG prepared from either non-immune sheep serum or antiserum to rabbit collagenase (V436/4). Non-immune IgG had no effect on either enzymic activity; the immune IgG decreased the collagenolytic activity to 14% of the control value whereas gelatinase activity remained at 81% of the control value.

Immunoinhibition of rabbit synovial fibroblast collagenase. The quantitation of inhibition of purified collagenase by IgG from sheep anti-(rabbit collagenase) antisera was further examined. Collagenase was mixed with various concentrations of sheep IgG from non-specific antiserum (X311/4), specific antisera (A3/2, V436/2, V436/4), antiserum to rabbit cathepsin D (D483/2), and normal sheep serum. As shown in Fig. 1, the anti-collagenase antisera were all inhibitory in the assay with reconstituted collagen fibrils whereas there was no inhibition of enzymic activity by IgG from non-immune serum or anti-(cathepsin D) antiserum. The non-specific antiserum X311/4 was the most inhibitory and gave the strongest precipitin lines in double immunodiffusion; the antiserum also contained about four times the normal concentration of IgG in serum.

The effect of immune IgG on the action of collagenase on collagen in solution at 35°C was also

Fig. 1. *Immunoinhibition of rabbit fibroblast collagenase*

Collagenase (0.045 unit/tube) was mixed with various concentrations of sheep IgG prepared from the following: a non-specific antiserum to rabbit collagenase, X311/4 (∇); specific antisera to rabbit collagenases, V436/4 ($\bullet$), V436/2 ($\blacktriangle$) and A3/2 ($\blacksquare$) respectively; an antiserum to rabbit cathepsin D, D438/2 ($\triangle$); and non-immune sheep serum ($\circ$). Fibril assays were for 18 h at 37°C .

Fig. 2. Inhibition of the action of collagenase on collagen in solution by sheep antiserum to rabbit fibroblast collagenase

Purified rabbit fibroblast collagenase ($0.8\mu\text{g}$) was mixed with either $167\mu\text{g}$ of IgG isolated from a specific antiserum to rabbit collagenase, V436/4 (Δ) or $146\mu\text{g}$ of IgG from non-immune sheep serum ($\bullet$). The mixtures were incubated at 35°C and the effect of the IgG preparations on the action of collagenase on collagen in solution was followed viscometrically; the viscosity of collagen incubated with IgG without enzyme is also shown ($\circ$).

tested. The steric hindrance of IgG binding to the enzyme would be less with collagen in solution and since this assay measures single clips through the triple helix of the collagen molecule it is a much more sensitive indicator of collagenase activity. As shown in Fig. 2, IgG prepared from a specific antiserum to rabbit collagenase diminished the rate of decrease of the viscosity of rabbit collagen by rabbit collagenase to about one-quarter of the rate in the presence of non-immune IgG. At this enzyme/immune IgG ratio there was residual enzymic activity demonstrable in the assay with radioactive fibrils.

Another test of the mechanism of inhibition of collagenase activity by antibodies was made by comparing the inhibitory capacity of immune IgG with Fab' prepared from it. The Fab' fragment was only slightly less inhibitory (mol/mol), on the basis of number of antibody-binding sites, than the parent IgG (Fig. 3). Hence inhibition does not depend on steric hindrance due to the cross-linking of the antigen-antibody complexes by multivalent antibodies.

Relationship between immunoinhibition and immuno-precipitation in gels

The strength of the precipitin line formed in double diffusion in gels and the inhibitory capacity on collagenase of the IgG isolated from the various antisera to collagenase were qualitatively related. As shown in Plates 2(a) and 2(b), the more inhibitory antisera (X311/4, V436/4) gave stronger precipitin lines than the less inhibitory antisera (V436/2, A3/2). Several other antisera are shown, and again their inhibitory capacity was related to their precipitating capacity in gels, under conditions of precipitation near equivalence.

Collagenase did not have to be enzymically active to react with the specific antiserum. Collagenase inactivated with EDTA reacted with the antiserum as did enzyme in the presence of Ca^{2+} , although the effect of Ca^{2+} on the interaction has not been examined systematically. This is different from bacterial collagenase, where in the absence of Ca^{2+} an antiserum raised against the Ca^{2+} -activated enzyme does not precipitate the enzyme (Soru & Zaharia, 1974).

Studies of immunoprecipitation in solution

The nature of the reaction of the anti-collagenase antibodies with collagenase was further examined by quantitative immunoprecipitation. Immunoprecipitate formed with the specific antiserum V436/4 correlated with the disappearance of enzymic activity (Fig. 4). One noteworthy observation was that the

Fig. 3. Comparison of the immunoinhibition of rabbit collagenase by IgG and fragment Fab'

Collagenase (0.051unit/tube) was mixed with various concentrations of the following: sheep IgG from a non-immune sheep ($\circ$); IgG from a specific antiserum to rabbit collagenase (V436/4) either as purified IgG ($\bullet$) or as a $(\text{NH}_4)_2\text{SO}_4$ precipitate ($\blacksquare$); Fab' fragments prepared from the IgG of the non-immune sheep (Δ); and Fab' fragments prepared from the IgG of antiserum V436/4 ($\blacktriangle$). Fibril assays were for 16h at 37°C .

Fig. 4. Relationship between immunoinhibition and immunoprecipitation of collagenase by a specific sheep antiserum to rabbit collagenase

Purified collagenase (6.2 units) was mixed with various amounts of IgG from either a non-immune serum (▲, △) or a specific antiserum to collagenase, V436/4 (●, ○), in 50 mM-Tris-HCl buffer, pH 7.6, containing 200 mM-NaCl and 5 mM-CaCl₂ (final vol. 0.5 ml). Precipitates were allowed to form overnight at room temperature. The tubes were centrifuged at 10000g for 10 min and then the collagenase activity in 25 µl portions of the supernatant (▲, ○) was determined by the reconstituted-collagen-fibril assay. Protein contents of the immunoprecipitates (▲, ●) were determined after solubilization in 1 M-NaOH.

precipitate dissolved in excess of antibody. In a similar experiment with various enzyme concentrations it was shown that the immunoprecipitate could also dissolve in excess of enzyme, with only a narrow zone where precipitation occurs. This has been observed for other antisera raised in sheep (Lazarus & Dingle, 1974), and consequently these antisera are not suitable for quantitatively precipitating collagenase molecules from solution.

The presence of immunoinhibited collagenase in immunoprecipitates and in soluble immune complexes made with the specific antiserum V436/4 was tested by assay for collagenolytic activity in the presence of 5 M-urea. By using insoluble bovine tendon collagen as substrate, about 20% of the activity could be recovered. The free enzyme was not stable to 5 M-urea, unless stabilized by protein, such as non-immune IgG. Gel electrophoresis at pH 3.5 of the fragments obtained after digestion of the insoluble cross-linked collagen with the enzyme-antibody complexes in the presence of 5 M-urea showed the formation of the specific α^A and α^B fragments.

Immuno-electrophoretic assay of collagenase

The nature of antisera to rabbit collagenase was studied by incorporating the antiserum into gel and

electrophoresing antigen into the gel by the method of Laurell (1972). Three noteworthy points derived from these studies. First, because of the narrow range of precipitation found with the sheep antisera, no 'rockets' could be seen with less than 4% (v/v) serum incorporated into the gels and therefore this technique was not a sensitive method for detecting collagenase. This problem has also been observed for horse antibodies, as compared with rabbit antibodies (Axelson *et al.*, 1973). Sheep antibodies appear to be intermediate between the highly precipitating rabbit antibodies and the poorly precipitating horse antibodies. The height of the precipitates formed in the 'rockets' was proportional to the amount of enzyme activity placed in the wells both for a set of standards of purified enzyme and for samples of collagenase derived from various rabbit tissues (Plate 2e). Because the isoionic point of collagenase was close to that of electrophoresis, the 'rockets' in some cases formed both anodally and cathodally. The method was an extremely sensitive method of discerning nonspecificity of antisera; when a range of IgG concentrations of up to 10% (v/v) of the serum concentration was used additional 'rockets' could be seen in some non-specific antisera. The highly nonspecific antiserum X311/4, gave eight or nine discernible 'rockets' (Plate 2f) compared with four or five lines (see Plate 2a) usually seen in double immunodiffusion. The specific antiserum V436/4 gave only a single 'rocket' at 10% (v/v) antiserum in gel with either crude, partially purified, or purified collagenase preparations.

Discussion

The present paper describes the production, characterization and properties of a specific antiserum of high avidity to collagenase purified from the culture medium of rabbit synovial fibroblasts (Werb & Reynolds, 1975). Before discussing the results and their implications, however, it is necessary to define what is meant by a specific antiserum. Much confusion arises when antisera are used for cytochemical and other studies without defining adequately their specificity: since there is no general agreement on definitions we have chosen a set of criteria which we consider to be adequate. First, in double immunodiffusion in gels by the method of Ouchterlony (1967), a supposed specific antiserum had to produce only one precipitin line with either purified collagenase or impure enzyme (concentrated culture medium), when both the antiserum and antigen were varied over tenfold ranges of concentration. This test had to hold true when the plates were run at either pH 8 or 6, and when Ca²⁺ was either present or absent. Additionally the development of the plates was observed by dark-ground

illumination at 24, 48 and 72h, and then stained to define any very weak line. Secondly, only one line had to be produced against impure enzyme on either immunoelectrophoresis or in 'rocket' assays. Although in our hands the 'rocket' assay technique was a sensitive method for defining the specificity of the antiserum it was not a very sensitive assay procedure for collagenase; it would perhaps be worthwhile to try the more precipitating guinea-pig antibodies in developing a sensitive 'rocket' assay. The two criteria just mentioned are generally applicable, but in the case of an antiserum to rabbit collagenase we applied the additional criteria that the antiserum would not show precipitin lines against collagen, against contaminants isolated during the purification of the enzyme, and against either rabbit serum or foetal calf serum (used for cell culture). We did not systematically test for non-precipitating antibodies because of the great difficulty in defining such interactions with possible contaminants and because there was usually good correspondence between the immunoinhibition and immunoprecipitation experiments.

Attempts to purify rabbit collagenase to such an extent that we could directly raise a specific antiserum were unsuccessful (Werb & Reynolds, 1975), so we adopted a combination of procedures that would purify the antibody and antigen. A novel procedure both to raise the first generation antiserum and to further purify the enzyme was to prepare α_2 -macroglobulin-complexes between the enzyme preparation and α_2 -macroglobulin derived from the sheep that was used to prepare the antiserum subsequently. Although the α_2 -macroglobulin-collagenase complex was unable to react with the antiserum raised, immunologically reactive material was found after destruction of the complex with NaSCN. This suggests that the immune system is capable of dissociating and/or recognizing foreign materials trapped within the α_2 -macroglobulin molecules. Such antisera were nonspecific and it seems likely that this is at least partly due to other proteinases having formed α_2 -macroglobulin-complexes (Werb *et al.*, 1974; Starkey & Barrett, 1973). However, the antisera produced by such a procedure were strongly inhibitory for rabbit collagenase, and produced good precipitin lines with purified enzyme that could be used as antigen in another sheep. Only 2–4 μ g of collagenase protein was present in each precipitin line so that only 50–100 μ g of enzyme was needed for the complete injection schedule for a sheep; antibody-antigen complexes appear to be about as immunogenic on the basis of protein as the equivalent amount of antigen alone (Weston, 1969). This procedure took two generations of precipitin lines to arrive at a specific antiserum and it seems unlikely that one can completely separate in one step the major precipitin line (collagenase-antibody) from other

minor contaminating lines and trace amounts of contaminants in gels.

All the immune IgG preparations, whether specific or nonspecific, were inhibitory in enzymic assays of collagenase (Table 2) and a good correspondence was usually obtained between the degree of inhibition and the intensity of precipitin lines in a gel. The specific antisera inhibited the action of rabbit collagenase in both fibril assays and with collagen in solution, showing that the configuration of the substrate, compared with that of the enzyme, was not important. It is also noteworthy that the inhibition occurred either with rat or with rabbit collagen, and that the action of rabbit collagenase on insoluble collagen can also be inhibited by the specific antisera (Z. Werb & J. J. Reynolds, unpublished results).

The antisera could inhibit the collagenase activity specifically without inhibiting the neutral proteinase activity (Werb & Reynolds, 1974), and this fact distinguishes the antisera from all other chemical inhibitors of metallo-proteinases (Werb & Burleigh, 1974). Although Werb & Burleigh (1974) found that most of the chemical inhibitors of collagenase were not effective below about 10 μ M, we estimated that we obtained equivalent inhibition with IgG between 10 and 0.1 nM (for the antiserum X311/4). In fact the inhibitory power of the antibody in our antisera must be greater than this because much of the IgG in the antiserum is not directed against collagenase. Since the enzyme does not have to be precipitated to be enzymically inactivated (see Cinader, 1967) it seems likely that only a few molecules of IgG are needed to block the enzymic activity of rabbit collagenase. It is also of interest that fragment Fab' is inhibitory, since the fragments of IgG are much more effective at penetrating tissues. This fact is important not only for understanding the inhibition but also for future cytochemical work and biological studies. The specific antiserum to rabbit fibroblast collagenase should prove to be a powerful tool in elucidating the biological role of collagenase in connective tissue remodelling.

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