Rabbit Collagenase

IMMUNOLOGICAL IDENTITY OF THE ENZYMES RELEASED FROM CELLS AND TISSUES IN NORMAL AND PATHOLOGICAL CONDITIONS

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1. The immunological cross-reactivity between rabbit collagenases from a variety of normal and pathological sources was examined. The specific antibody raised against collagenase secreted from normal rabbit synovial fibroblasts gave reactions of complete identity with collagenases secreted from fibroblasts derived from rabbit skin, and from synovium from experimentally arthritic rabbits. 2. The rabbit fibroblast collagenase was immunologically identical with collagenases obtained from the organ culture medium of normal rabbit skin, synovium, ear fibrocartilage and subchondral bone. 3. Collagenases from the culture media of normal rabbit synovium and from hyperplastic synovium of rabbits made experimentally arthritic were identical. 4. The collagenase secreted from rabbit fibroblasts gave a reaction completely identical with that of a collagenase extracted directly from a rabbit carcinoma. 5. IgG (immunoglobulin G) from a specific antiserum to rabbit fibroblast collagenase was a potent inhibitor of the collagenases obtained from the culture media of the various rabbit cells and tissues. 6. Collagenases from human synovium and from mouse macrophages and bone were neither precipitated nor inhibited by antibodies to rabbit collagenase. 7. No immunoreactive material was found in lysates of rabbit polymorphonuclear leucocyte granules with the specific antisera to rabbit fibroblast collagenase. No evidence for inactive forms of rabbit collagenase in lysates of the rabbit synovial fibroblasts could be found, either by double immunodiffusion against the specific collagenase, or by displacement of active enzyme from inhibition by the IgG.

Since the first description of a vertebrate collagenase (Gross & Lapiere, 1962) capable of degrading undenatured collagen, many collagenases have been isolated from a variety of normal and diseased tissues (for reviews, see Harris & Krane, 1974*a,b,c*). To clarify the cell biology and biochemistry of the synthesis and secretion of these enzymes in physiological and pathological conditions it is very important to establish the structural relationship between the collagenases from various sources. So far, reports in the literature have suggested that there may be several forms of the enzyme in some tissues (Bauer *et al.*, 1970, 1971; Harris *et al.*, 1969) and that different tissues in the same species may produce collagenases of differing molecular weights (Woolley *et al.*, 1973); also mammalian collagenases have been reported to have mol.wts. as high as 158 000 (for reviews, see Harris & Krane, 1974*a,b,c*; Nagai, 1973). The work described in the present paper is an attempt to try to resolve some of the problems on the interrelationships between collagenases by examining collagenases produced by

a variety of tissues from rabbits. The rabbit was chosen as the species for investigation because we have prepared a specific antiserum to the collagenase secreted by rabbit synovial fibroblasts maintained in culture (Werb & Reynolds, 1975*a,b*). The data in the present paper establish the immunological identity of collagenases produced by a variety of rabbit cells and tissues.

Materials and Methods

The immunochemical methods used were as described by Werb & Reynolds (1975*a,b*).

Rabbit fibroblast lines were established from explants of normal rabbit synovium and skin, and synovium from rabbits made experimentally arthritic as described previously (Werb & Burleigh, 1974). Conditioned media, used as the source of collagenase, were prepared from secreting cell lines by culturing newly confluent fibroblast monolayers in Dulbecco–Eagle's medium in the absence of serum for 72 h as described by Werb & Burleigh (1974) and

Werb & Reynolds (1975a). Tissue collagenases from rabbit ear cartilage, skin, normal synovium, experimentally arthritic synovium and articular cartilage with small amounts of attached subchondral bone, were obtained by culturing small pieces of tissue in Dulbecco-Eagle's medium without serum for 10 days, with medium changes every 2 days essentially as described by Werb & Burleigh (1974). Medium containing collagenase activity was concentrated by ultrafiltration before use.

Rabbit heterophil leucocytes were obtained from peritoneal exudates, and granule preparations were made as described by Cohn & Hirsch (1960). Purified and crude collagenase preparations extracted from the rabbit V2 ascites tumour grown intramuscularly (Harris *et al.*, 1972) were the gift of Dr. E. D. Harris, Jr., Dartmouth Medical School, Hanover, N.H., U.S.A.

Human rheumatoid synovium was provided by Mr. A. Murley and his colleagues, Orthopaedic Surgery Department, Addenbrookes Hospital, Cambridge, U.K. Human rheumatoid synovial collagenase was prepared by culturing human synovium in Dulbecco-Eagle's medium, as described for rabbit synovium (Werb & Burleigh, 1974). Human synovial fluid collagenase was the preparation described previously (Werb *et al.*, 1974). Collagenase was obtained from the conditioned culture medium of thioglycollate-stimulated mouse macrophages (Unkless *et al.*, 1974) and the characterization of this enzyme is described elsewhere (Werb & Gordon, 1975).

Immune-enzyme displacement assays

These assays were made by the principles described by McGee & Undenfriend (1972), in which inactive enzyme forms are estimated by the displacement of active enzyme from inhibitory antibody. Studies with rabbit collagenases were made by using IgG (immunoglobulin G) isolated from the specific antiserum, V436/4 (Werb & Reynolds, 1975b). Antisera were designated by the number of the animal followed by the number of the bleed (Werb & Reynolds, 1975b). Purified collagenase was used in sufficient quantities to give approx. 60% lysis of reconstituted radioactive rabbit collagen fibrils in 18 h at 37°C (approx. 0.05 unit/tube). The unit of collagenase activity was defined by Werb & Reynolds (1975a). The immune IgG preparations used were treated for immunoinhibition as described by Werb & Reynolds (1975b) and an inhibitory concentration was chosen which was on the linear part of the sigmoidal dose-response curve. Usually 20% of the enzymic activity remained with approx. 100 µg of the immune IgG. Appropriate controls with non-immune IgG were made similarly. Six assays for enzymic activity were made in the assay with radioactive collagen fibrils for each sample

to be tested for the presence of displacing antigen. First, the sample was assayed for endogenous collagenase activity and second, was tested for non-specific inhibitory material by mixing with purified collagenase. Third and fourth, either the immune or the non-immune IgG was mixed with the sample to see if activity appeared. Finally for the fifth and sixth assays, the sample was mixed with purified collagenase in the presence either of the immune or of non-immune IgG. The appearance of collagenase activity over the amount present from the residual-plus-endogenous collagenase activity of the sample would constitute a positive result. Samples that inhibited collagenase activity non-specifically could not be measured by this technique.

Other methods and materials were as described by Werb & Reynolds (1975a,b).

Results

Secretion of collagenases from various rabbit tissues in culture

A variety of rabbit tissues were cultivated and the collagenolytic activity in their culture fluids was measured to obtain rabbit collagenases with which to make an immunochemical comparison with rabbit synovial fibroblast collagenase. As shown in Table 1, skin, arthritic synovium and articular cartilage with subchondral bone released collagenase into the medium, with high activities being measurable at day 2, and these secretory rates were sustained for several days; ear cartilage and normal synovium secreted little activity initially, but this activity

Table 1. *Secretion of collagenase from various rabbit tissues in culture*

Tissues excised from rabbits were cultured in Dulbecco's modification of Eagle's medium, with daily medium changes as described by Werb & Burleigh (1974). Wet weights of tissues were determined at the start of the experiment. One unit of collagenase hydrolyses 1 µg of collagen/min (Werb & Reynolds, 1975a).

Tissue	Collagenase activity in medium (units/24h per g of tissue)	
	Day 2	Day 5
Skin	4.0	3.1
Ear cartilage	0.4	2.1
Articular cartilage	0	0
Articular cartilage+ subchondral bone	8.7	5.5
Normal synovium	0.4	2.3
Experimentally arthritic synovium	4.1	4.8

EXPLANATION OF PLATE I

Immunodiffusion plates

(a) Cross-reaction between collagenases secreted from rabbit cells. As, Specific antiserum to rabbit fibroblast collagenase (V436/4). Wells contained concentrated culture medium from (1) normal rabbit synovial fibroblasts, (2) rabbit skin fibroblasts, (3) rabbit fibroblasts from synovium from animals with experimentally induced arthritis, (4) purified rabbit synovial-fibroblast collagenase, (5) rabbit skin collagen, (6) concentrated culture medium from rabbit synovial fibroblasts secreting no enzymically detectable collagenase. (b) Immunological reactivity of rabbit synovial fibroblasts and their conditioned culture medium. Wells contained (1) specific antiserum to rabbit fibroblast collagenase (V436/4), (2) non-specific antiserum to rabbit collagenase (A3/4), (3) culture medium from rabbit synovial fibroblasts (concentrated to 1 ml, from 3×10^6 cells grown for 72h), (4) cell lysate (3×10^6 fibroblasts/ml of 0.1% Triton X-100). (c) Cross-reaction between collagenases secreted from rabbit fibroblasts and tissues. As, Specific antiserum to rabbit fibroblast collagenase (V436/4). Wells contained concentrated culture medium from (1) experimentally arthritic synovium, (2) normal synovial fibroblasts, (3) normal synovium, (4) fibroblasts from experimentally arthritic synovium. (d) Cross-reaction between collagenases from various rabbit tissues. As, Specific antiserum to rabbit fibroblast collagenase (V436/4). Wells contained concentrated culture medium from (1) normal synovium, (2) normal synovial fibroblasts, (3) skin fibroblasts, (4) skin, (5) ear cartilage, (6) articular cartilage with subchondral bone. (e) Cross-reactivity of rabbit fibroblast collagenase and rabbit tumour collagenase. Wells contained (1) concentrated collagenase from normal rabbit synovial fibroblasts, (2) extract of rabbit tumour, (3) nonspecific antiserum to rabbit fibroblast collagenase (X311/4), (4) specific antiserum to rabbit fibroblast collagenase (V436/4). (f) Reaction of antiserum with rabbit heterophil leucocyte granules. Wells contained (1) rabbit polymorphonuclear leucocyte granule extract, (2) antiserum to rabbit fibroblast collagenase (V436/4), (3) antiserum to rabbit cathepsin D (A339/2). (g) Immunological relationship between rabbit fibroblasts which do or do not secrete enzymically active collagenase. Wells contained (1) medium from rabbit fibroblasts not secreting collagenase, concentrated 100 times, (2) medium from secreting fibroblasts, concentrated 100 times, (3) non-specific antiserum to rabbit collagenase (Z10/3), (4) specific antiserum to rabbit collagenase (V436/4), (5) mixture of Z10/3 and antiserum to bovine serum proteins.

Table 2. *Immunoinhibition of various collagenases by sheep anti-(rabbit synovial fibroblast collagenase) IgG*

IgG was prepared from sheep antisera to rabbit collagenase (V436/4), rabbit cathepsin D (A339/2) and non-immune sheep serum (NSS), and tested for ability to inhibit the activity of collagenases from various sources in the assay with reconstituted collagen fibrils. The IgG (50–200 µg/tube) was mixed with the collagenase preparation, and then incubated with the collagen for 18 h at 37°C. n.d., Not determined.

Collagenase source	IgG	...	Enzymic activity (% of control)		
			V436/4	NSS	A339/2
Rabbit culture media					
Synovial fibroblasts			0	106	98
Skin fibroblasts			3	103	105
Skin			5	100	n.d.
Subchondral bone			0	98	n.d.
Arthritic synovium			5	100	n.d.
Normal synovium			0	107	n.d.
Ear cartilage			8	94	n.d.
Rabbit V2 tumour extract			0	100	n.d.
Human rheumatoid synovium culture medium			96	105	n.d.
Human rheumatoid synovial fluid			110	101	n.d.
Mouse culture media (thiogly- collate-stimulated macrophages)			102	105	98

increased with time. Articular cartilage alone secreted no detectable collagenase activity.

Inhibition of the enzymic activity of various collagenases by sheep anti-(rabbit synovial fibroblast collagenase) antiserum

The extent of immunological cross-reactivity of collagenases from various rabbit sources and other species was first analysed by assessing the inhibition by immune IgG of enzymic activity in the assay with radioactive collagen fibrils. The collagenases were all in the form either of crude concentrated conditioned tissue-culture medium or of crude extracts. As shown in Table 2, all the rabbit tissues were completely inhibited by IgG purified from the sheep anti-(rabbit fibroblast collagenase) antiserum, whereas collagenase obtained from human and mouse sources were unaffected. Non-immune IgG, and IgG from an antiserum directed against rabbit cathepsin D were not inhibitory. These findings strongly suggest that there are considerable structural similarities between the rabbit collagenases.

Studies of immunoprecipitation in gels

Immunological identity of collagenases from various rabbit cells. The specific antiserum to rabbit synovial fibroblast collagenase showed a reaction completely identical with collagenases from the culture medium

of fibroblast lines derived from explants of rabbit skin, and from synovium of experimentally arthritic rabbits (Plate 1a). Immunologically reactive material could not be demonstrated in culture medium from rabbit synovial fibroblasts that did not secrete enzymically detectable collagenase activity, and there was no cross-reaction with collagen extracted from rabbit skin. Lysates of rabbit synovial fibroblasts did not show any immunologically reactive material when tested in double diffusion against the specific antiserum V436/4 to the collagenase secreted from the cells (Plate 1b), or with a slightly non-specific antiserum (A3/4), although precipitin lines were obtained with a very non-specific antiserum (X311/4).

Immunological identity of collagenases from rabbit tissues. Collagenases secreted into the culture medium of rabbit skin, ear fibrocartilage, normal synovium, synovium from experimentally arthritic rabbits, and articular cartilage with attached subchondral bone, were immunologically identical with the rabbit fibroblast collagenase (Plate 1c and 1d).

Immunological identity between rabbit fibroblast collagenase and collagenase extracted from a rabbit tumour. The extent of cross-reactivity between collagenase secreted from normal fibroblasts (and other normal tissues in culture) and the collagenase extracted from a solid ascites tumour growing in rabbit muscle (Harris *et al.*, 1972) was next examined. Both crude and purified tumour enzyme reacted with antiserum raised against the fibroblast collagenase. As shown in Plate 1(e), a completely identical reaction was found between the fibroblast and tumour enzymes when tested with the specific antiserum V436/4, and crude tumour extracts gave multiple lines against a non-specific antiserum (X311/4). Further, IgG purified from the specific antiserum to rabbit collagenase (V436/4) was able to inhibit the activity of the tumour enzyme in the assay with radioactive fibrils (Table 2).

Studies with rabbit heterophil leucocytes. Rabbit polymorphonuclear leucocyte granules have been reported to contain collagenase activity (Robertson *et al.*, 1972). Although we were unable to detect significant enzymic activity in leucocyte granule preparations, the immunological cross-reactivity with the antiserum to rabbit fibroblast collagenase was examined (Plate 1f). No immunological reactivity was found between the specific antiserum to rabbit fibroblast collagenase (V436/4) and rabbit heterophil leucocyte granule lysates, although the granules gave a clear precipitation line in double diffusion against the antiserum to rabbit cathepsin D (D483/2). A single precipitin line was obtained between the granule lysates and the non-specific antisera (A3/4 and X311/4) to rabbit fibroblast collagenase, but this line did not appear to be identical with the specific immunoprecipitate with rabbit fibroblast collagenase.

Studies with collagenase from other species. Collagenases obtained from the culture medium of human synovium, mouse calvaria and mouse macrophages, and the rabbit fibroblast collagenase, were tested for immunological cross-reactivity by immunoprecipitation in gels with the specific antiserum to rabbit collagenase (Table 2). The mouse and human enzymes were not precipitated by the specific sheep anti-(rabbit collagenase) antiserum, and there was no inhibition of the enzymic activity of the human synovial collagenase or mouse macrophage collagenase demonstrable even by ten times the concentration of immune IgG giving complete inhibition of the rabbit collagenase.

Studies on inactive forms of rabbit collagenase. The antibody to collagenase was used to search for enzymically inactive precursors of collagenase. As mentioned above no precipitation reaction was obtained with lysates of rabbit synovial fibroblasts secreting collagenase activity into their extracellular milieu (Plate 1b). Accordingly the possibility of immunological cross-reaction without precipitation was examined by the technique of studying the displacement of active enzyme from inhibitory IgG by inactive forms (McGee & Udenfriend, 1972). Lysates of rabbit synovial fibroblasts had no detectable inactive precursor when tested by this method. Rabbit polymorphonuclear leucocyte granules also gave no evidence of cross-reactivity by this procedure.

Another application of this procedure was to examine whether rabbit fibroblasts that did not secrete collagenase activity were secreting an inactive form of the enzyme. Werb & Reynolds (1974) showed that different rabbit synovial fibroblast lines had different intrinsic rates of secretion of collagenase. We now have isolated cell lines that do not secrete active collagenase under normal culture conditions in serum-free medium. When concentrated, the medium from the non-secreting cells cross-reacted with non-specific antisera to collagenase but not with the specific antiserum (Plate 1g). When examined by immune enzyme displacement no immunologically reactive molecules were detectable. The sensitivity of the procedure would have detected 1/100 of the usual secretion of collagenase (1 unit/24 h per 1×10^6 cells). We conclude that precursors, if they exist, do not have cross-reacting precipitating antibodies with the active collagenase and do not react with IgG molecules to produce inhibition.

The enzyme- α_2 M-macroglobulin complexes, forms of collagenases that do not give precipitin lines with specific antisera to collagenase (Werb & Reynolds, 1975b), also did not compete for antibody molecules in the immune displacement assays.

Discussion

The present paper provides evidence that there is complete immunological identity between colla-

genases isolated from the culture medium of a variety of rabbit cells and tissues, and that all these enzyme preparations were inhibited by IgG from the specific antiserum to the collagenase from rabbit synovial fibroblasts. The establishment of this fact is important in order that the antiserum may be used in future studies as a specific probe to investigate the biochemistry and physiological role of collagenase *in vivo*. The specific antiserum used in these studies was raised against a collagenase purified from the culture medium of rabbit fibroblasts (Werb & Reynolds, 1975a,b). This antiserum was shown to inhibit the collagenase specifically among the proteinases secreted from rabbit fibroblasts (Werb & Reynolds, 1975b), and evidence in the present paper shows that the antiserum does not cross-react with collagenases either from human synovium or from mouse bone or macrophages.

All the above data strongly suggest that the collagenase produced by different rabbit tissues are very similar proteins. It seems that collagenases do not have tissue-specific forms, as do some enzymes (Sussman *et al.*, 1968; Sherwin *et al.*, 1969), and their relationship with each other is analogous to cathepsin D. Dingle *et al.* (1971) used specific antisera to cathepsin D from several species to show that there was immunological identity between enzyme preparations from different tissues, but not from different species. Dingle *et al.* (1971) also showed that isoenzymes of cathepsin D showed complete identity, and we do not rule out the possibility that there are isoenzyme forms of rabbit collagenase. The results of Bauer *et al.* (1970) and Eisen *et al.* (1974) with human and rat collagenases also point to the species differences rather than tissue differences, and it is noteworthy that although there is no immunological cross-reaction between collagenases from different species, they conserve such specialized enzymic activity (see Harris & Krane, 1974a,b,c). However, our antisera cannot give information on the chemical identity of the collagenase and only purification of the enzyme from different tissues will show if they have similar molecular weights. It seems possible that differences in molecular weight (see, e.g., Woolley *et al.*, 1973) may be due to the existence of dimer or trimer forms.

The specific antiserum showed that there was complete identity between the collagenase from rabbit synovial cells and from a rabbit tumour on the basis of both immunoinhibition and immunoprecipitation. These data suggest that the collagenase from the tumour may not be unique, as has been reported for other proteinases from tumours (see, e.g. Unkeless *et al.*, 1973), but it also raises the question as to whether the collagenase is secreted by the tumour cells or by surrounding host fibroblasts. This question, along with many others, can

only be answered by using the antiserum in immunocytochemical experiments to localize the enzyme. It is noteworthy that the collagenase from the culture medium of rabbit fibroblasts (Werb & Reynolds, 1975a) has a very similar mol.wt. (35000) to that of the collagenases isolated from the rabbit tumour (E. D. Harris, personal communication), and that of collagenase released from rabbit skin in culture (M. C. Burleigh, personal communication).

It has been proposed for mouse bone collagenase (Vaes, 1972) and tadpole collagenase (Harper *et al.*, 1971) that the enzyme is first synthesized as an enzymically inactive precursor that has been termed 'procollagenase' (see also Nagai, 1973). We could find no evidence for any inactive form of the rabbit collagenase from the synovial cells, either by double immunodiffusion of cell lysates against the specific antiserum, or by immunodisplacement experiments (McGee & Udenfriend, 1972). If such a precursor form exists for the rabbit collagenase we have investigated, then either it does not cross-react with the specific antiserum or it occurs at a low concentration compared with active enzyme. We could also find no material that reacted with the specific antiserum when concentrates of medium from cells producing no active enzyme were tested.

We have also tested lysates of rabbit polymorphonuclear granules with the specific antiserum and could find no cross-reacting material with the other rabbit collagenase, suggesting that this particular collagenase may be different (Robertson *et al.*, 1972). However, we could find little specific collagenase activity in the rabbit polymorphonuclear granules, and it seems possible that much of the activity of the rabbit granules against collagen may not be specific collagenase, but perhaps the lysosomal elastase (Burleigh, 1975), or the neutral proteinases described by Dewald *et al.* (1975). In addition, rabbit polymorphonuclear leucocytes contain only a small fraction of the amount of lysosomal elastase that is present in human granulocytes (Z. Werb, unpublished observation), and if this were also true for collagenase, then it might not be detectable by our present methods.

The data in this paper, together with our previous work (Werb & Reynolds, 1975a,b) demonstrate that our antiserum specifically precipitates and inhibits rabbit collagenases from a variety of sources, and could be used to localize the enzyme cytochemically in many experimental situations.

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