Collagenase is a Component of the Specific Granules of Human Neutrophil Leucocytes

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Azurophil and specific granules were isolated from human polymorphonuclear neutrophil leucocytes. Collagenase was almost exclusively a component of the specific granules. This finding is in contrast with the distribution of other proteolytic enzymes, which are localized in the azurophil (or lysosomal) granules.

Recent research has shown that collagenases active at neutral pH values play an essential role in the catabolism of collagenous tissues (Harris & Krane, 1974*a,b,c*). As indicated by tissue-culture experiments, collagenases are synthesized and secreted by many tissues, but are not usually stored intracellularly in appreciable amounts. Polymorphonuclear neutrophil leucocytes are a notable exception. In these cells collagenase has been found in the granule fraction (Lazarus *et al.*, 1968, 1972), and, like other granule components (Bainton *et al.*, 1971), is presumably synthesized and packaged into granules during maturation.

Two main types of granule have been identified morphologically and biochemically in human neutrophils (Bainton *et al.*, 1971; Bretz & Baggiolini, 1974; Dewald *et al.*, 1975). Azurophil granules contain a range of acid hydrolases found in lysosomes in other cells, lysozyme and neutral proteinases, such as elastase and cathepsin G. Specific granules contain lysozyme and lactoferrin but appear to lack acid hydrolases. In the present communication we provide evidence that collagenase is localized almost exclusively in the specific granules. Neutrophil infiltration into sites of inflammation is frequently associated with the degradation of structural proteins in connective tissues. The subcellular localization of collagenase, separate from the other proteinases, could provide a form of selective control of degradative enzyme release by differential discharge of granule types.

Methods

Preparation of azurophil- and specific-granule extracts

Human neutrophils were purified from peripheral blood, homogenized, fractionated by zonal sedi-

mentation and assayed for marker enzymes as described previously (Bretz & Baggiolini, 1974). Azurophil and specific granules were collected by pooling and concentrating appropriate fractions as indicated in Fig. 1.

Extracts were prepared by incubating the granule fractions [azurophils, 4.5mg of protein/ml, and specifics, 2.8mg of protein/ml (protein determined by the method of Miller, 1959)] for 2h in 200mm-sodium acetate, pH4.5, containing 10mm-CaCl₂, at 4°C (Ohlsson & Olsson, 1973). The incubation mixtures were then adjusted to pH7.6 with 2M-Tris and centrifuged at 30000*g* for 30min.

Characterization of collagenase activity

Extracts from granule fractions were incubated with purified rat skin collagen in solution and the products of digestion analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Reaction mixtures (0.3ml) contained 100μg of acid-soluble rat skin collagen (prepared by the method of Werb & Burleigh, 1974) in 50mm-Tris/HCl (pH7.6)/50mm-NaCl/10mm-CaCl₂; incubations were for 20h at 25°C. Where indicated, collagenase activation was carried out by preincubation of the extracts with trypsin (5μg/ml) at 25°C for 1h, followed by the addition of soya-bean trypsin inhibitor (25μg/ml). In some cases 4-chloromercuribenzoate (0.7mm final concentration) was included in the reaction mixture, as this increased activity (Lazarus *et al.*, 1972). Reaction mixtures were denatured by the addition of sodium dodecyl sulphate (final concentration 1%) followed by boiling for 1min before application to the gels. Full details of the electrophoresis and staining of gels are given elsewhere (Werb & Reynolds, 1975). The collagenolytic activity of elastase (Barrett, 1975) was abolished by the treatment of granule

extracts with 10mM-Dip-F (di-isopropyl phosphoro-fluoridate). This enabled collagenase activity to be identified (Fig. 1).

Results and Discussion

Two distinct populations of granules were separated and identified as azurophils and specifics by the marker-enzyme distribution (Fig. 1). The final pools of azurophil and specific granules represented 12.8 and 5.1% of the starting protein content respectively. The distribution of enzyme activities between the pools, as percentages of total activity, was as follows: (azurophils/specifics) lysozyme, 25.1:25.9; peroxidase, 52.0:5.2; elastase, 56.6:3.7; alkaline phosphatase, 8.9:7.6. Peroxidase and elastase, which are exclusive components of the azurophil granules (Bretz & Baggiolini, 1974; Dewald et al., 1975), are a measure of contamination of specifics by azurophils. The contamination of the azurophil granules by the specific granules cannot be assessed at present in human neutrophils because of the lack of a suitable biochemical marker. It is likely to be between 5 and 10% (see Baggiolini, 1974). Unpublished work (U. Bretz & M. Baggiolini) has indicated that alkaline
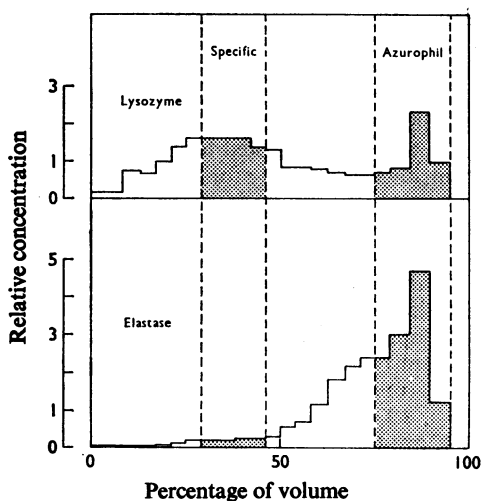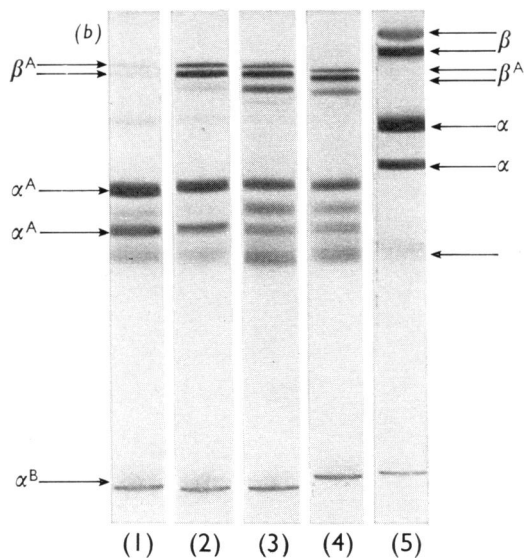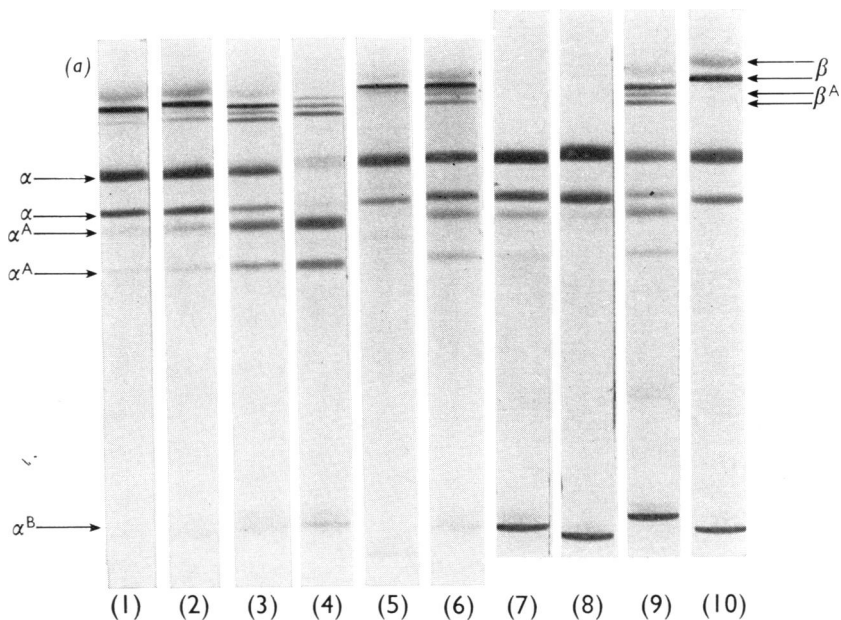
phosphatase arises from contamination of both granule preparations by plasma membranes.

In comparison with other collagenases, neutrophil collagenase has low activity on reconstituted collagen fibrils (Lazarus et al., 1972). The two granule extracts were therefore tested on collagen in solution by the described techniques. The specificity of the collageno-lytic activities was assessed by comparing the electrophoretic patterns of the collagen-degradation products with those produced by partially purified rabbit skin collagenase (Plate 1a). Both granule extracts showed collagenase activity, as indicated by the degradation of the β and α chains of collagen to the characteristic β^A and α^A fragments [Plate 1a; compare gels (1) and (4)]. However, the amount of extract needed to demonstrate activity was very much higher for the azurophil than for the specific-granule preparation (150μg as opposed to 1.8μg of extract protein; Plate 1a). β^A fragments were not seen in azurophil-granule incubations unless they were pre-incubated with Dip-F [Plate 1a; compare gels (7) and (9)]. Dip-F is an inhibitor of elastase, which is present in large amounts in these granules (Dewald et al., 1975) and is known to catalyse the β- and α-chain conversion (M. Burleigh, personal communication). Only with high concentrations of specific-granule extracts was the elastase contamination sufficient to demonstrate a Dip-F-inhibitable β- into -α conversion [Plate 1b; compare gels (1) and (2)]. More typically, the fragments produced by the specific-granule extracts were identical with those produced by the reference collagenase [Plate 1a; gel (6)]; this was confirmed by electrophoresis of admixtures of the respective reaction products (not shown).

We conclude that the neutrophil collagenase cleaves collagen into the typical three-quarter and one-quarter fragments, as suggested by others (Lazarus et al., 1968, 1972; Kruze & Wojtecka, 1972; Ohlsson & Olsson, 1973), and like other collagenases it is completely inhibited by EDTA [Plate 1b; gel (5)]. By use of viscometric assays (Werb & Reynolds, 1975) we estimate that the collagenase activity is approx. 150 μg/min per mg of specific-granule protein.

Plate 1(b) shows that further fragments of collagen, in addition to the usual β^A and α^A products, are obtained on incubation with high concentrations of the specific-granule extract. Like collagenase this additional collagenolytic activity is inhibited by EDTA, is not inhibited by Dip-F and is strongly enhanced by treatment of the granule extracts with trypsin and 4-chloromercuribenzoate.

Our results show that human neutrophil collagenase is largely associated with the specific granules. The low collagenase activity of our azurophil preparation could easily be accounted for by contaminating specific granules. Indeed, more recently we have obtained an azurophil fraction without any collagenase activity. Collagenase has been reported by

Fig. 1. *Separation of azurophil and specific granules from human neutrophils by zonal differential sedimentation* Human neutrophils were purified from buffy coats of peripheral blood to 97% purity. A total of 2×10^9 cells were homogenized and fractionated by zonal sedimentation through a discontinuous gradient of 450–800mM-sucrose (50mM steps) in a B-XIV rotor at 10500rev./min for 15min (Bretz & Baggiolini, 1974). The fractions were characterized by determination of lysozyme and elastase as marker enzymes. Preparations of azurophil and specific granules were collected by pooling (stippled areas) and subsequent centrifugation at $3 \times 10^6 g$-min.

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the denatured products of digestion of collagen by extracts of azurophil- and specific-granule preparations under various conditions

Each gel contained the equivalent of about $10\,\mu g$ of collagen. Under the conditions of these experiments the smaller α^B fragments ran at or near the Bromophenol Blue/buffer front. α refers to the single polypeptide chains of collagen and β to the cross-linked dimers of the α chains. Collagenase clips the entire triple helix of native collagen at a single point to produce three-quarter-length β^A and α^A, and one-quarter-length α^B pieces. At higher concentrations of specific-granule protein there is a faint protein band which runs similarly to that of one of the collagen fragments (arrow). (*a*) The reaction mixtures included: for gels (1)–(4), 1.8, 3.6, 9 and $18\,\mu g$ of protein respectively from specific-granule preparations; for gel (5), buffer alone; for gel (6), 0.07 unit of rabbit skin collagenase (Werb & Burleigh, 1974); for gel (7), $150\,\mu g$ of azurophil-granule protein; for gel (8), $150\,\mu g$ of azurophil-granule protein and EDTA (10mM); for gel (9), $150\,\mu g$ of azurophil-granule protein treated with Dip-F and 4-chloromercuribenzoate; for gel (10), $150\,\mu g$ of azurophil-granule protein treated with Dip-F, 4-chloromercuribenzoate and EDTA. (*b*) The reaction mixtures included: for gel (1), $45\,\mu g$ of specific-granule protein; for gel (2), $45\,\mu g$ of specific-granule protein treated with Dip-F and 4-chloromercuribenzoate; for gel (3), $45\,\mu g$ of trypsin-activated specific-granule protein in the presence of 4-chloromercuribenzoate; for gel (4), $45\,\mu g$ of trypsin-activated specific-granule protein treated with Dip-F and 4-chloromercuribenzoate; for gel (5), $45\,\mu g$ of trypsin-activated specific-granule protein treated with Dip-F, 4-chloromercuribenzoate and EDTA.

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Robertson *et al.* (1972) to be localized in specific granules of rabbit neutrophils. However, our subcellular localization of human neutrophil collagenase is in disagreement with a preliminary report by Ohlsson (1975) that collagenase and elastase are both in the azurophil granules. The compartmentation of collagenase in the specific granules may explain why it is apparently released from phagocytosing neutrophils with a different time-course from that of other neutral proteinases and acid hydrolases (Oronsky *et al.*, 1973). The specific granules have been shown to discharge into phagocytic vacuoles more readily than do azurophils (Bainton, 1973; Brederoo & Daems, 1970), and, under special experimental conditions, specific-granule contents may be exocytosed (Weissmann *et al.*, 1975; Hoffstein, 1976).

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