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SOME PROPERTIES OF HUMAN BLOOD MONOCYTE CELL LYSATE NEUTRAL PROTEINASE(S)

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The proteinase content of highly purified preparations of human peripheral blood monocytes was investigated. Monocyte cell lysates exhibited activity at neutral pH against azocasein, ^{3}H-labelled elastin as well as several synthetic substrates used to detect serine proteinases (EC 3.4.21.-) of human polymorphonuclear leucocytes. The cell lysates also contain at least two acid proteinases. The levels of neutral proteinase activity in monocytes was considerably less than that found in polymorphonuclear leucocytes. The effect of inhibitors on the monocyte neutral proteinases showed them to be of the serine type. Monocytes also solubilized and degraded the type IV collagen found in human glomerular basement membrane at neutral and acid pH. The action of the monocyte proteinase on glomerular basement membrane indicated that their properties were similar but not identical to that of the polymorphonuclear leucocyte serine proteinases. Since monocytes infiltrate the glomerulus in certain forms of immunologically mediated glomerulonephritis, it may well be that monocyte serine proteinases make a contribution to the glomerular damage that occurs.

Introduction

It is becoming increasingly evident that monocytes and macrophages play an essential role in a number of chronic inflammatory processes [1,2]. Morphologic and cultural techniques suggest that these cells may be involved in the pathophysiology of experimental and clinical glomerulonephritis [3–7]. In certain types of glomerulonephritis, infiltrating polymorphonuclear leucocytes can destroy glomerular basement membrane by degradation of the collagen and other structural glycoproteins [8,9]. Monocytes and macrophages contain, synthesize and secrete a variety of enzymes active at acid or neutral pH [10]. The relationship of these enzymes to glomerular basement membrane damage in immunologically mediated glomerulonephritis is unknown. Macrophage proteintases active at neutral pH have been suspected to initiate the sequence of degradative processes of cartilage proteoglycans associated with rheumatoid arthritis [11]. The same proteinases could possibly initiate glomerular basement damage in glomerulonephritis.

Werb and Gordon [12,13] showed that mouse peritoneal macrophages secrete a neutral collagenase and an elastase, and Hauser and Vaes [14] have demonstrated the degradation of cartilage by a neutral proteinase secreted by rabbit-bone-marrow macrophages in culture. The neutral proteinases of human monocytes are less well studied. Ragsdale and Arend [15] have described a plasminogen-independent fibrinolytic enzyme in human monocytes in culture which has many of the features of leucocyte elastase. The purpose of the present study is to report on the proteolytic activity of human peripheral blood monocytes. In this paper we confirm that human monocytes contain elastase-type proteinases and

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid. Z-Ala-2-ONap, *N*-benzyloxycarbonyl-L-alanine 2-naphthyl ester; Bz-DL-Phe-2-ONap, *N*-benzoyl-DL-phenylalanine 2-naphthyl ester; Bz-DL-Arg-2NNap, *N*-benzoyl-DL-arginine 2-naphthylamide; Bz-DL-Arg-Nan, *N*-benzoyl-DL-arginine 4-nitroanilide; Boc-Ala-ONp, *t*-butyloxycarbonyl-L-alanine 4-nitrophenyl ester; Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester.

present some evidence for their involvement in glomerulonephritis.

Part of the work described has been presented in preliminary form [16,17].

Materials and Methods

Materials

Dulbecco's modified Eagle's medium with Hepes buffer and 0.85 g/l $NaHCO_3$ was purchased from Flow Laboratories; Ficol was from Pharmacia and Hypaque from Winthrop Laboratories, New York. Glass beads (60 mesh) were purchased from British Drug Houses and were washed in nitric acid and rinsed extensively in distilled water prior to use.

Human glomerular basement membrane was prepared from post mortem kidneys by the method of Krakhower and Greenspon [18] as detailed by Davies et al. [8].

Preparation of human peripheral monocytes

Human monocytes were prepared from 100 ml heparinized blood taken with consent, by centrifugation on a Ficol-Hypaque gradient to remove polymorphonuclear leucocytes and subsequent elution from glass beads to separate the lymphocytes from the monocytes [19]. The purity of the preparation was determined by the combined esterase demonstration as described by Stuart et al. [20] and the Giemsa stain.

Assays

Activity against azocasein. Neutral proteolytic activity was measured with azocasein as substrate at pH 7.5 and 50°C essentially as described by Starkey [21]. A unit of activity is defined as that amount which hydrolyzes 1 mg azocasein per h.

Activity against [^{3}H]acetylated casein. [^{3}H]-Acetylated casein was obtained as described by Klinman and Karush [22]. Equal volumes of the labelled casein solution (10 mg casein/ml, 260 000 dpm/mg casein) and cell lysates were incubated at 37°C in a final volume of 50 µl. The reaction was stopped by the addition of 250 µl 11.5% (w/v) trichloroacetic acid and the suspension was cooled in an ice-bath for 30 min prior to centrifugation. An aliquot of the supernatant was taken and counted. 1 unit of activity refers to the amount of enzyme that degrades 1 µg casein into trichloroacetic acid-soluble fragments per min.

Activity against [^{3}H]elastin. Elastinolytic activity was measured with [^{3}H]elastin as described by Starkey [21].

Activity against naphthol esters. Activity against Z-Ala-2-ONap and Bz-DL-Phe-2-ONap, substrates used for human lysosomal elastase and cathepsin G, respectively, were carried out as described by Starkey [21].

Gel chromatography. The digestion products from the incubation of glomerular basement membrane with human blood monocytes were fractionated by gel filtration at 4°C in a column (1.5 × 90 cm) of Sephadex G-200 using an eluting buffer of 50 mM K_2HPO_4/KH_2PO_4 (pH 7.4) containing 0.1 M NaCl. Protein was monitored by absorbance at 280 nm and 3-ml fractions were examined for hydroxyproline using the technique described by Woessner [23] as modified by Burleigh et al. [24] with internal standards. Proteins were assumed to have an $E^{1\%}_{280}$ (1 cm lightpath) of 10.0. Calibration of the column was achieved by elution of the following compounds under identical conditions: Blue Dextran (V_0), bovine serum albumin (Va; M_r 68 000), cathepsin B prepared from human kidney (Vb; M_r 26 000) and cytochrome *c* (Vc; M_r 13 000).

Results

Separation of human lymphocytes, polymorphonuclear leucocytes and monocytes

In all, ten preparations from human peripheral blood were examined which contained greater than 95% monocytes as judged by the nonspecific esterase stain. Less than 1% were polymorphonuclear leucocytes. Preparations of monocytes containing more than 1% polymorphonuclear leucocytes were rejected. The proteolytic activity of cell lysates prepared from pure preparations of lymphocytes, monocytes and polymorphonuclear leucocytes is shown in Table I. Lymphocytes contained no neutral proteinase activity. These cells did contain proteolytic activity at acid pH, probably due to cathepsin D. There was no activity against Bz-DL-Arg-2-NNap and the lymphocyte cell lysate contained no detectable inhibitor of a purified preparation of cathepsin B. As expected, polymorphonuclear leucocytes contain

TABLE I

NEUTRAL AND ACID PROTEINASE ACTIVITY OF HUMAN BLOOD MONOCYTES, LYMPHOCYTES AND POLYMORPHONUCLEAR LEUCOCYTES

The neutral proteinase activity of cell lysates of pure preparations of human monocytes, lymphocytes and polymorphonuclear leucocytes was determined with azocasein. Acid proteinase activity was assayed with haemoglobin at pH 3.4 (cathepsin D) and Bz-DL-Arg-2NNap at pH 6.0 (cathepsin B). The lysates were prepared by sonication of the appropriate cell in a solution containing 1 M NaCl, 2% (v/v) propan-2-ol, and 1 mM EDTA. Each value represents the mean of duplicate assays after subtraction of blank values. 1 unit of neutral proteinase activity is expressed as the hydrolysis of 1 mg azocasein per h. 1 unit of cathepsin D is the ΔE_{280} of 1.0/h. 1 unit of cathepsin B is that activity hydrolysing 1 nmol substrate per s (nkat). Results are expressed for 10^9 cells. Values are given the mean ± S.D. Number of determinations in parenthesis; n.d., not detected.

Enzyme	Monocytes	Lymphocytes	Polymorphonuclear leucocytes
Neutral proteinase	95 ± 43 (5)	n.d.	1223 ± 150 (9)
Acid proteinase (cathepsin D)	13.6 ± 2.2 (3)	84.6 ± 20.7 (4)	35.7 ± 3.1 (9)
Cathepsin B	3.8 ± 1.5 (3)	n.d.	n.d.

considerable quantities of several proteinases active against azocasein at neutral pH [21]. The monocyte cell lysate also contained activity against azocasein at neutral pH but very much smaller amounts than polymorphonuclear leucocytes. On a cellular basis the polymorphonuclear leucocytes contained over 12-fold the neutral proteinase content of monocytes.

Properties of monocyte cell lysate neutral proteinase: effect of pH, time and enzyme concentration

A more convenient and sensitive method of assaying neutral proteinase activity is to utilize [^{3}H]-acetylated casein as substrate. The use of this substrate is particularly valuable for the study of monocyte cell lysates because of the low levels of neutral proteinase activity found in these cells.

The enzymic degradation of [^{3}H]acetylated casein as followed by the release of trichloroacetic acid soluble radioactivity with 0.33 unit of neutral proteinase (units of azocasein activity) as a function of time is shown in Fig. 1a and is linear with incubation period of up to 360 min. The results shown in Fig. 1b indicate that the release of trichloroacetic acid-soluble radioactivity was linear with cell lysate concentration up to 1 unit of neutral proteinase activity. The pH-dependence of the enzyme was also assessed with [^{3}H]acetylated casein using sodium formate and potassium phosphate buffers (Fig. 2). Optimal activity was observed over a neutral pH range of 6.8–8.2.

Enzyme inhibition studies

Table II shows the effect of various agents on the neutral proteinase activity of human blood monocytes. Phenylmethanesulphonyl fluoride produced total inhibition, whereas the thiol reagent cysteine and EDTA had very little effect. Soybean trypsin inhibitor also gave powerful inhibition of the monocyte enzyme. Human serum, as expected, was shown to be a potent inhibitor. The above results suggest that the neutral proteinase activity is largely due to a serine proteinase.

The hydrolysis of haemoglobin by monocyte cell lysates at pH 3.4 was completely inhibited by pepstatin (1 μg/ml). The known cathepsin B inhibitors leupeptin (1 μM) and 4-chloromercuribenzoate (0.1 mM) produced total inhibition of the activity against Bz-DL-Arg-2NNap. Thiol-dependent degradation of azocasein at pH 6.0 was also inhibited by the mercury salt.

Substrate specificity of the monocyte neutral proteinase

(a) Activity against chromogenic substrates. The monocyte cell lysate was inactive against Bz-DL-Arg-Nan, a substrate for trypsin. At pH 7.5, however, there was activity against two synthetic substrates of lysosomal elastase (Z-Ala-2-ONap and Boc-Ala-ONp). Furthermore, the lysate hydrolsed Bz-DL-Phe-2ONp and Bz-Tyr-OEt, chymotrypsin substrates which are

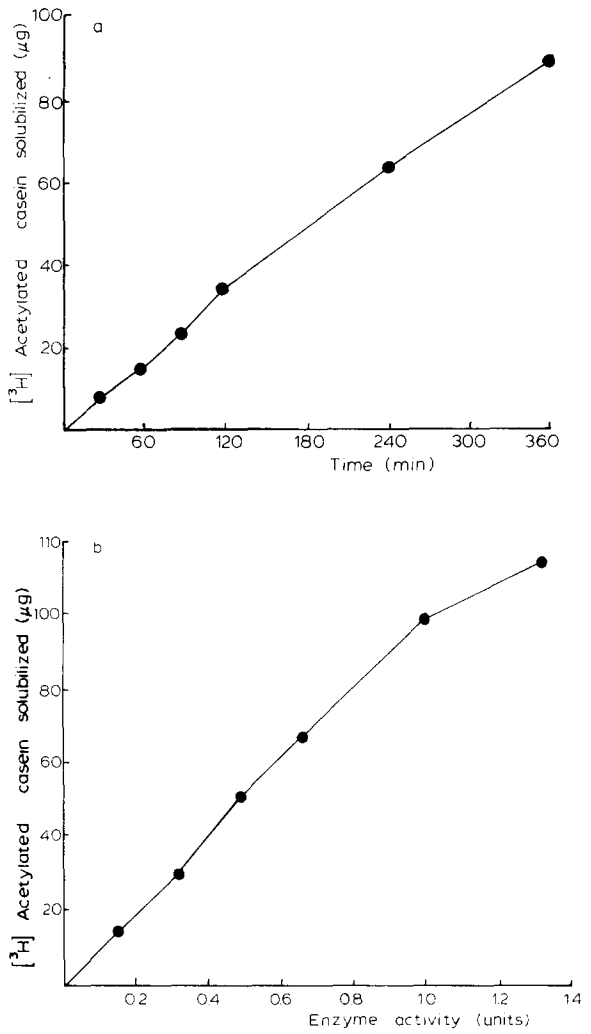

Fig. 1. Linearity with time (a) and enzyme concentration (b) of release of radioactive peptides soluble in trichloroacetic acid from [^{3}H]acetylated casein. Human blood monocyte lysate (0.33 neutral proteinase unit) was incubated at 37°C for the time intervals indicated. In b human monocyte lysates (0.16–1.25 units neutral proteinase activity) were incubated at 37°C in a final volume of 100 μl for 2 h.

both hydrolysed by cathepsin G [21].

(b) Activity against macromolecules. In addition to their activity against the above synthetic substrates for lysosomal elastase and cathepsin G, the blood monocyte lysates also hydrolysed azocasein, [^{3}H]-elastin and glomerular basement membrane.

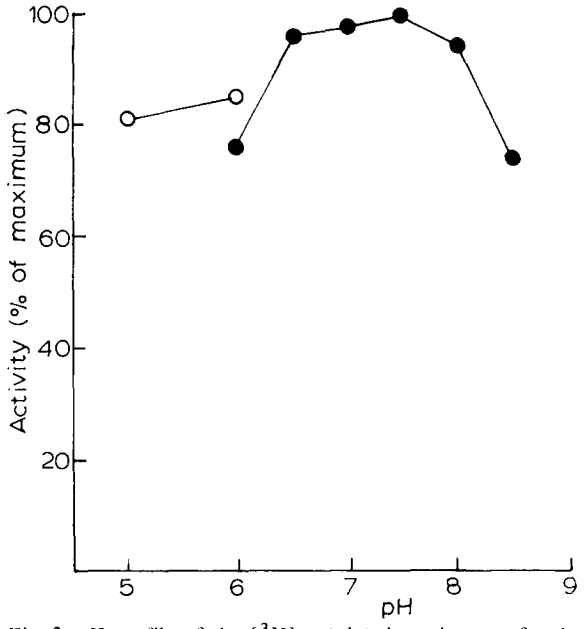

Fig. 2. pH profile of the [^{3}H]acetylated casein assay for the neutral proteinase of human blood monocytes. Monocyte lysate (50 μl) containing 0.7 unit neutral proteinase activity against azocasein was added to each tube and 200 mM buffer to give a final concentration of 100 mM. Incubation was at 37°C for 2 h. Buffers used were: sodium formate (○) and potassium phosphate (●). Values are expressed as a percentage of the maximum activity.

Release of hydroxyproline from human glomerular basement membrane

The monocyte cell lysates were found capable of solubilizing glomerular basement membrane as measured by the release of hydroxyproline-containing proteins and peptides into the incubation medium. The pH activity profile for this activity is shown in Fig. 3 and is very different to that for the degradation of glomerular basement membrane by a granule extract prepared from human polymorphonuclear leucocytes. The degradation at pH 3.5 was not inhibited by pepstatin (1 μg/ml), neither was it inhibited by leupeptin (100 μM). In an earlier publication we have shown that highly purified cathepsin B but not cathepsin D extensively degrades glomerular basement membrane [8]. The inability of leupeptin to prevent glomerular basement membrane degradation by the monocyte cell lysate at acid pH is, therefore, of interest. At neutral pH the degradation of glomerular basement membrane is completely

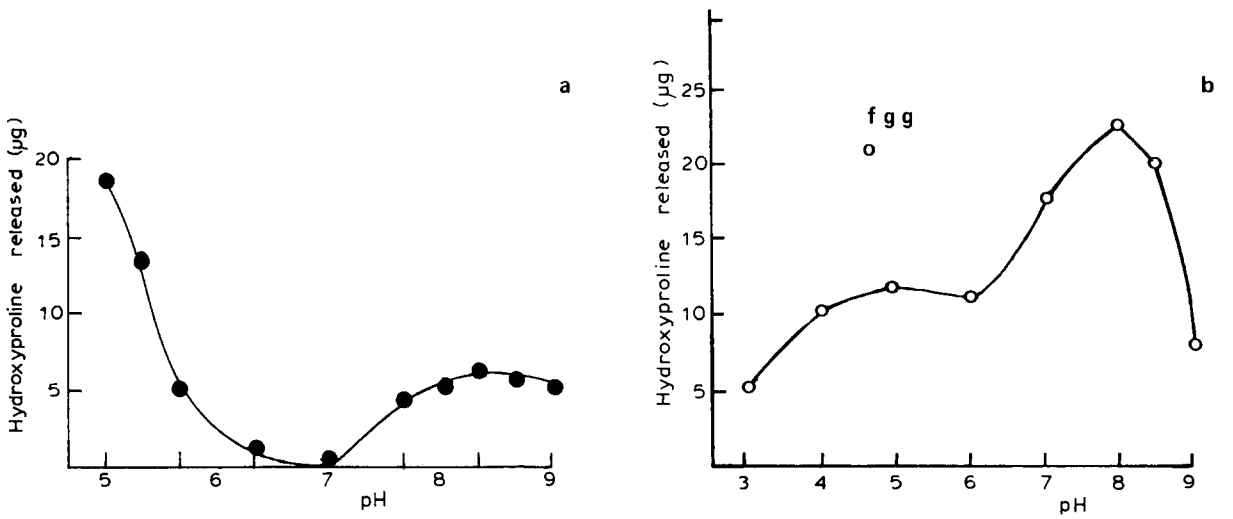

Fig. 3. Effect of pH on the degradation of human glomerular basement membrane by (a) human blood monocyte lysate and (b) human polymorphonuclear leucocyte. Reaction mixtures containing in (a) 0.05 units of neutral proteinase activity and in (b) 2.78 units of neutral proteinase activity were incubated with 2 mg glomerular basement membrane in 100 mM buffer in a total volume of 0.5 ml at 37°C for 20 h. Buffers used were sodium formate, acetate and potassium phosphate. The results are expressed in µg hydroxyproline released per mg glomerular basement membrane.

inhibited by phenylmethanesulphonyl fluoride and soybean trypsin inhibitor (Table II).

The rate of degradation of glomerular basement membrane by monocyte cell lysates, expressed as µg hydroxyproline released per h per unit of neutral proteinase activity, was 14.99 ± 7.4 (S.D.) (n = 6). In corresponding experiments with polymorphonuclear leucocytes the rate was 0.99 ± 0.26 (S.D.) (n = 5). When these results are expressed on a cell basis they indicate that glomerular basement membrane degrading activity of monocytes is slightly higher than that of polymorphonuclear leucocytes. The result is noteworthy in view of the high neutral proteinase activity of polymorphonuclear leucocytes as measured with azocasein as substrate (see Table I).

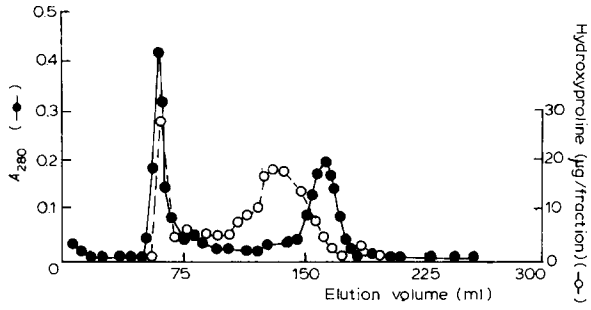

Fig. 4. Gel filtration of the soluble products from the digestion of insoluble glomerular basement membrane by a cell lysate of human blood monocytes. 20 mg glomerular basement membrane were incubated with monocyte lysate (1.6 units neutral proteinase activity measured against azocasein) in 0.1 M potassium phosphate buffer, pH 7.4, in a final volume of 4 ml. After 64 h the incubation mixture was centrifuged for 12 000 g · min at 4°C. 3 ml of the clear supernatant were applied immediately to Sephadex G-200 and eluted from the column. Fractions were collected and each was examined for protein (•———•) and hydroxyproline (o———o).

Characterization of glomerular basement membrane degradation products

The material made soluble after incubation of glomerular basement membrane with human monocyte lysates was examined by gel filtration on Sephadex G-200. Fig. 4 shows the distribution of protein and peptides together with that of hydroxyproline-containing material. Two peaks of hydroxyproline-containing material are observed. The first has a molecular weight of approx. 180 000 and is eluted immediately after the void volume. The second emerges between bovine serum albumin (M_r 68 000) and human cathepsin B (M_r 26 000).

TABLE II

EFFECT OF VARIOUS AGENTS AND SERUM PROTEINS ON MONOCYTE CELL LYSATE NEUTRAL PROTEINASE ACTIVITY

The various agents marked [a] were dissolved in propan-2-ol, before being diluted into the incubation mixture. The final concentration of the solvent was 4% (v/v). All other inhibitors were in aqueous solution. Enzyme activity is expressed as a percentage of that activity produced by the cell lysate alone. Assays were of proteolytic activity against (a) [^{3}H]acetylated casein and (b) glomerular basement membrane. n.d., not done.

Reagent added	Concentration in assay	Activity (% control)	
		a	b
EDTA	5 mM	97.0	100.2
1,10-Phenanthroline	1 mM	96.0	99.0
Cysteine	5 mM	110.0	n.d.
Phenylmethanesulphonyl fluoride [a]	1 mM	0	0
Soybean trypsin inhibitor	1 mg/ml	3.0	0
$CaCl_2$	5 mM	87.0	n.d.
4-Chloromercuribenzoate	0.1 mM	64.0	n.d.
Human serum proteins	2% (v/v)	2.0	0
1-Chloro-4-phenyl-3-tosylamido-L-butan-2-one (TPCK) [a]	1 mM	90.0	n.d.

Discussion

In this paper we have shown that human peripheral blood monocytes contain neutral proteinase activity which has several properties similar to lysosomal elastase and cathepsin G found in human polymorphonuclear leucocytes [21]. Critical to our observations, therefore, is the identification of the monocyte as the sole source of the neutral proteinase activity in our final preparations and not polymorphonuclear leucocytes which contain high levels of neutral proteinases (see Table I). To establish the purity of the monocyte preparations we relied on the characteristic feature of monocytes to stain for non-specific esterase activity which gives monocytes a multiple red granular appearance in the cytoplasm [20]. Correlation of the monocyte count using this stain with other histological stains also aided the establishment of the purity of our preparation. Polymorphonuclear leucocytes constituted less than 1% of the total cell count. The final preparation also contained up to 5% lymphocytes.

In addition to histological criteria there is considerable biochemical evidence to support our claim that the monocyte is the source of the neutral proteinase. Firstly, highly purified preparations of lymphocytes showed no neutral proteinase content. Furthermore, the lymphocyte preparation contained no inhibitory component effect against polymorphonuclear leucocyte proteinase or the monocyte lysate activity against azocasein. These results effectively rule out any contribution from the lymphocytes. Secondly, from consideration of the data in Table I it can be calculated that if the neutral proteinase activity of the monocyte preparations resulted from polymorphonuclear leucocytes then it would require at least 7% contamination. Thirdly, the monocyte neutral proteinase activity can be distinguished from the polymorphonuclear leucocytes by its ability to degrade glomerular basement membrane. Finally, monocytes, but not lymphocytes or polymorphonuclear leucocytes, contain activity at pH 6.0 towards Bz-DL-Arg-2NNap – a substrate for cathepsin B.

Several reports have appeared of neutral proteinases in macrophages derived from different species. Mouse peritoneal macrophages under certain conditions contain a neutral proteinase active against elastin but not against azocasein or the low molecular weight substrates commonly used to detect elastase activity [12,13]. This enzyme is inhibited by EDTA and phenylmethanesulphonyl fluoride but not by soybean trypsin inhibitor. Vaes and his co-workers [14] have described a metal-dependent neutral proteinase in rabbit bone marrow macrophages that

degrades the protein core of proteoglycan subunits and which is distinct from other neutral proteinases secreted from mouse or guinea-pig macrophage. Rabbit alveolar macrophages secrete a similar enzyme [25]. Human macrophages are less well documented. Preliminary observations by Werb and Gordon [13] suggested human blood monocyte (blood macrophages) in culture secrete an elastase similar in properties to that of the mouse peritoneal macrophages. In contrast, Ragsdale and Arend [15] have demonstrated that human blood monocytes incubated on surface-bound immune complexes secrete a plasminogen-independent fibrinolytic enzyme with properties characteristic of polymorphonuclear leucocyte lysosomal elastase but not derived from lymphocytes or contaminating polymorphonuclear leucocytes. The neutral proteinases described in the present work appear similar in several respects to the plasminogen-independent fibrinolytic enzyme. Janoff and his coworkers [26] have also reported that human alveolar macrophages contain small amounts of an elastin-degrading enzyme which exhibited less activity towards Ac-$(Ala)_3$-OMe, an elastase substrate, than either commercial pancreatic elastase or polymorphonuclear leucocyte elastase. Macrophages also contain or secrete neutral collagenase [10]. We did not determine whether human monocytes contain such a proteinase, but the cell lysates did release soluble hydroxyproline-containing peptides from glomerular basement membrane. Since this attack on type IV collagen was inhibited by phenylmethanesulphonyl fluoride and soybean trypsin inhibitor but not by EDTA it is likely that a serine-type proteinase and not a metal-dependent neutral proteinase was responsible. It has already been reported that polymorphonuclear leucocyte elastase and cathepsin G degrade glomerular basement membrane. In contrast to the polymorphonuclear leucocyte enzymes, the monocyte neutral proteinases were rather more selective against the basement membrane. It is not known whether this is due to the specificity of monocyte serine proteinases or to the presence of one or more as yet uncharacterized enzymes. Several reports have appeared of elastase activity in different species, which indicate that each has different specificity towards inhibitors and substrates [21].

The inability of leupeptin to inhibit the degradation of glomerular basement membrane at acid pH deserves comment. It is clear that highly purified preparations of human liver cathepsin B have the capacity to degrade interstitial collagen as well as the type IV collagen of basement membrane [8,24]. In contrast to the action of cathepsin B, cathepsin D does not attack collagen or glomerular basement membrane. The identity of the acid proteinase responsible for glomerular basement degradation in the present work is, therefore, unknown. A possible candidate is a collagenolytic cathepsin. Collagenolytic cathepsins have been described in a variety of mammalian tissues including rat granulation tissue [27], rat post partum uterus [28], bovine spleen [29] and rabbit leucocytes [30]. These are characterized by a low pH optimum, activation by thiol compounds and the ability to degrade polymerized collagen.

Recently it has been suggested that collagenolytic cathepsins may be identical with cathepsin L [31]. However, cathepsin L is strongly inhibited by leupeptin, which argues against the idea that collagenolytic cathepsin is the acid proteinase responsible for the degradation of glomerular basement membrane in the present investigation. Further characterization of this enzyme is required.

The observation that mononuclear cell lysate degrades glomerular basement membrane could be important for the understanding of the pathophysiology of certain types of glomerular disease. Infiltrating mononuclear cells have been described in diseased glomeruli of antiglomerular basement membrane nephritis in rat and rabbit [3,4] as well as in experimental immune complex disease [7]. Their presence has also been shown by morphological and culture techniques in the human disease [6]. Our results are preliminary but provide indirect evidence that monocytes can play a role in glomerulonephritis through the action of their enzymes on glomerular basement membrane.

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