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Granulocyte collagenase: Selective digestion of type I relative to type III collagen

(neutrophil/fibroblast/tissue inflammation/connective tissue)

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ABSTRACT Collagenases produced by human polymorphonuclear leukocytes, human lung fibroblasts, and rabbit pulmonary alveolar macrophages were compared in their ability to digest soluble native type I and type III collagens. While the fibroblast and macrophage collagenases attacked the two substrates at approximately equal rates, the leukocyte collagenase attacked type I collagen preferentially (15:1) in comparison to type III collagen. This was true with human or rabbit collagen substrates. Thus, proteolysis of collagen, particularly in acute inflammation, may have a significant role in controlling the types of collagen present in connective tissue.

The interstitial structure of many tissues includes two distinct collagens, termed types I and III (1, 2). Not only is the relative proportion of the two types different in different tissues (3), but the ratio of type I to type III in the same tissues has been found to vary during development (2, 4, 5), in certain diseases (6, 7), and in inflammation and repair (8–10). This is of general importance to organ function, because the relative proportion of the two collagen types appears to have a major influence on tissue mechanical properties. For example, in pliable, compliant tissues (aorta, spleen, lung, small intestine), there are significant proportions of type III collagen, while in rigid tissues (bone, tendon) there is only type I (3). With age, the I/III ratio of human skin markedly increases (2), with concomitant decreases in skin compliance. In diseases associated with fibrosis [e.g., atherosclerosis (6), pulmonary fibrosis (7)], there is a shift in the balance of collagen types, resulting in an increase in the proportion of type I; this change is accompanied by a loss of tissue compliance and normal organ function.

The amount of collagen found in any organ depends on a balance of collagen synthesis and degradation. Under the physiologic conditions of the extracellular milieu, the latter is attributed to the action of collagenases, enzymes that specifically attack native collagen (11). Although collagen synthesis is presumed to be important in the control of the relative amounts of type I and type III collagen found in a tissue (4, 5), collagenase is not, since in all cases studied, collagenases have been found to degrade these two collagen types at the same rate (12, 13). Thus, even if tissues were to change the amount of collagenase available to attack collagen, there is, as yet, no evidence that the relative proportion of collagen types I and III would alter.

However, during the inflammatory process, the cellular composition of an organ changes, in part due to the appearance, first, of polymorphonuclear leukocytes and, later, mononuclear cells. In this report, we present data suggesting that the collagenase from acute phase inflammatory cells (polymorphonuclear leukocytes) is relatively ineffective in digesting type III collagen, while the collagenase from tissue cells (fibroblasts) and late stage inflammatory cells (macrophages) digests type I and type III collagen equally well, thus suggesting an important mechanism for controlling the composition of the extracellular matrix in disease.

MATERIALS AND METHODS

Preparation of Collagenases. Human blood polymorphonuclear leukocytes (PMN) were isolated by sedimentation through Hypaque–Ficoll followed by dextran sedimentation (14, 15). Over 99.5% of the isolated cells were PMN and the preparations were free of platelets and mononuclear cells as judged by stained smears. The PMN were washed with phosphate-buffered saline and plated (2 to 5 × 10^5 cells per cm^2) on plastic tissue culture dishes in serum-free Dulbecco's modified Eagle's medium. After 24 hr, the medium was collected, concentrated 50-fold by dialysis against Aquacide II (Calbiochem), and dialyzed against buffer for the collagenase assay (50 mM Tris·HCl at pH 7.4, 5 mM $CaCl_2$, 200 mM NaCl).

Human lung fibroblast collagenase was prepared from the medium of confluent monolayers of diploid human fetal lung fibroblasts (HFL-1) derived from a 16-week-old fetus. Medium from subcultivation 10 at cell density 10^7/100 mm plate was concentrated 50-fold by dialysis against Aquacide II and dialyzed against the buffer for the collagenase assay. Under these conditions, all of the fibroblast collagenase was in a latent form and it was necessary to activate the enzyme with trypsin by a modification of the method of Bauer *et al.* (16). Trypsin (100 μg/ml; twice crystallized, Sigma) was added to the latent enzyme and the mixture was incubated at 25° for 20 min. The trypsin was then inactivated by the addition of soybean trypsin inhibitor (500 μg/ml) and the active enzyme was dialyzed against the buffer for the collagenase assay. Trypsin activity (17) was absent from the final collagenase preparation.

Rabbit pulmonary alveolar macrophages were isolated by lavage of lungs of an inbred strain of adult New Zealand White rabbits (B and H Rabbitry, Rockville, Md.) and the cells were plated as previously described (18). Medium (modified Eagle's medium without serum) between 24 and 48 hr in culture was collected, concentrated by dialysis against Aquacide II, and dialyzed against buffer for the collagenase assay.

All enzymes were stored at −195° without loss of activity for greater than 6 months.

Preparation of Substrates. All collagenase assays were performed using labeled soluble native collagen substrates prepared from confluent monolayers of either newborn rabbit lung fibroblasts (NB-6) or fetal human lung fibroblasts (HFL-1) in subcultivation 15 (NB-6) or 10 (HFL-1). The cells were incubated in serum-free modified Eagle's medium with [^{3}H]proline (31 Ci/mmol, 50 μCi/ml), β-aminopropionitrile (0.5 mM), and ascorbic acid (0.3 mM) for 24 hr and radioactive type I procollagen and type III procollagen were isolated from the medium using diethylaminoethyl-cellulose chromatography

Abbreviation: PMN, polymorphonuclear leukocytes.

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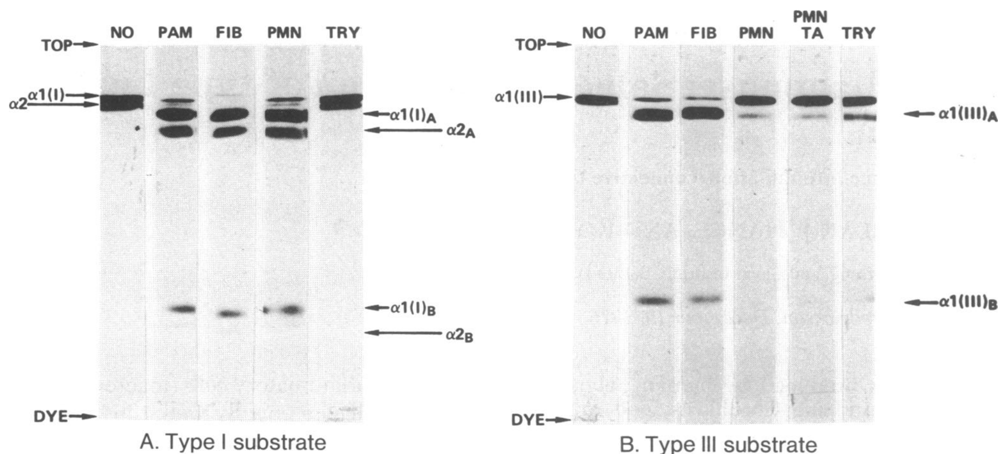

FIG. 1. Fluororadiogram of sodium dodecyl sulfate/acrylamide gel electrophoresis of rabbit type I [^{3}H]collagen and type III [^{3}H]collagen incubated with rabbit alveolar macrophage, human lung fibroblast, and human polymorphonuclear leukocyte collagenases. (*A*) Rabbit type I collagen substrate. Each incubation contained 15×10^4 dpm of rabbit [^{3}H]proline-labeled type I collagen in 50 μl together with no enzyme (NO), 0.28 μg of pulmonary alveolar macrophage collagenase (PAM), 0.13 μg fibroblast collagenase (FIB), 0.98 μg of polymorphonuclear leukocyte collagenase (PMN), or 5 μg trypsin (TRY). The intact $\alpha1$(I) and $\alpha2$ chains and the $\alpha1(\mathrm{I})_A$, $\alpha2_A$, $\alpha1(\mathrm{I})_B$, and $\alpha2_B$ collagenase digestion products of the type I substrate are indicated. (*B*) Rabbit type III collagen substrate. Each incubation was identical as with the type I substrate. In addition, the type III substrate was incubated with 0.98 μg of PMN collagenase after pretreatment of the collagenase with trypsin (PMN-TA) (2 μg/ml trypsin for 20 min at 25° followed by the addition of 10 μg/ml soybean trypsin inhibitor). Intact $\alpha1$(III) chains and the $\alpha1(\mathrm{III})_A$ and $\alpha1(\mathrm{III})_B$ collagenase digestion products of the type III substrate are indicated.

(19). The labeled procollagens were dialyzed against 0.5 M acetic acid, converted to type I and type III collagen with limited pepsin digestion (100 μg/ml, 4°, 16 hr), and dialyzed against the buffer for the collagenase assay. Labeled rabbit and human collagens prepared in this matter were 100% pure type I and >98% pure type III, respectively, based on sodium dodecyl sulfate/acrylamide gel electrophoresis and cyanogen bromide peptide analysis (19). The final [^{3}H]proline-labeled collagen substrates had the following specific activities: rabbit type I, 5.9×10^5 dpm/μg; rabbit type III, 5.5×10^5 dpm/μg; human type I, 5.1×10^4 dpm/μg; and human type III, 4.9×10^4 dpm/μg.

The sedimentation velocity profiles of the collagen substrates were determined with sucrose gradient analysis (5–20% sucrose gradients in 50 mM Tris·HCl at pH 7.4, 5 mM $CaCl_2$, 200 mM NaCl). Each sample, in the same buffer, was layered on a gradient and centrifugation was carried out in an SW 41 rotor (Beckman Instruments) at 40,000 rpm for 22 hr at 24°. Following centrifugation, 0.3 ml fractions were collected and their radioactivities were measured in Aquasol (New England Nuclear) with an efficiency of 30%. Standards of chymotrypsinogen ($s_{20,w}$ = 2.6 S) and bovine serum albumin ($s_{20,w}$ = 4.2 S) were detected by measurement of absorbance at 280 nm.

Collagenase Assays. All assays were carried out in 50 μl final volume in 50 mM Tris·HCl at pH 7.4, 5 mM $CaCl_2$, 200 mM NaCl for 14 hr at 24° using 15×10^4 dpm rabbit type I, 15×10^4 dpm rabbit type III, 18×10^4 dpm human type I, or 18×10^4 human type III as substrates. Following incubation, 15 μl of the incubation mixture was heated to 60° for 30 min in the presence of 1% sodium dodecyl sulfate/0.1% 2-mercaptoethanol and then subjected to sodium dodecyl sulfate/acrylamide slab gel electrophoresis (200 V, 3.5 hr, 10% acrylamide/0.1% bisacrylamide with a 2.5% acrylamide/0.625% bisacrylamide stacking gel) using a discontinuous buffer system (20). Following electrophoresis, the gels were dried and fluororadiograms were prepared. Under the conditions used, the band densities of the fluororadiograms were proportional to the amount of radioactivity present (21). Quantitative values for the bands representing labeled material were determined by densitometry using a Gilson spectrophotometer equipped with a scanning device.

The following protease inhibitors were used to evaluate the collagenases: ethylenediaminetetraacetate, dithiothreitol, phenylmethylsulfonylfluoride, *N*-ethylmaleimide, bovine trypsin–kallikrein inhibitor (Aprotinin, Sigma), bovine serum albumin, human serum, and human $\alpha1$-antitrypsin (Worthington).

RESULTS

Under the conditions used, rabbit type I collagen [composed of two $\alpha1$(I) chains and one $\alpha2$ chain] was digested by human fibroblast, human PMN, and rabbit macrophage collagenase into specific fragments designated $\alpha1(\mathrm{I})_A$ and $\alpha(\mathrm{I})_B$ for the $\alpha1$(I) chain and $\alpha2_A$ and $\alpha2_B$ for the $\alpha2$ chain. As expected for vertebrate collagenases, these fragments are approximately 75% ("A" fragment) and 25% ("B" fragment) of the original molecule (11). Rabbit type I collagen was not susceptible to attack by trypsin (Fig. 1*A*). Similar results were found using human type I collagen as the substrate (Fig. 2*A*). As reported for other vertebrate collagenases, the attack of type I collagen by fibroblast, PMN, and macrophage collagenases could be inhibited by ethylenediaminetetraacetate, dithiothreitol, and human serum, but not by phenylmethylsulfonylfluoride, *N*-ethylmaleimide, bovine trypsin–kallikrein inhibitor, bovine serum albumin, or human $\alpha1$-antitrypsin (Table 1) (11).

However, when type III collagen was used as the substrate, there were striking differences between PMN collagenase and fibroblast or macrophage collagenase. Under the conditions used, it appears that human fibroblast and rabbit macrophage collagenases easily digest type III collagen into the expected $\alpha1(\mathrm{III})_A$ and $\alpha1(\mathrm{III})_B$ pieces. In comparison, the same amounts of PMN collagenase that readily digested the type I rabbit substrate barely cleaved the type III rabbit substrate (Fig. 1*B*). This was not because some of the PMN collagenase was in a latent form, since attempts to activate the PMN collagenase with trypsin gave the identical result. However, in contrast to rabbit type I substrate, the rabbit type III collagen was partially

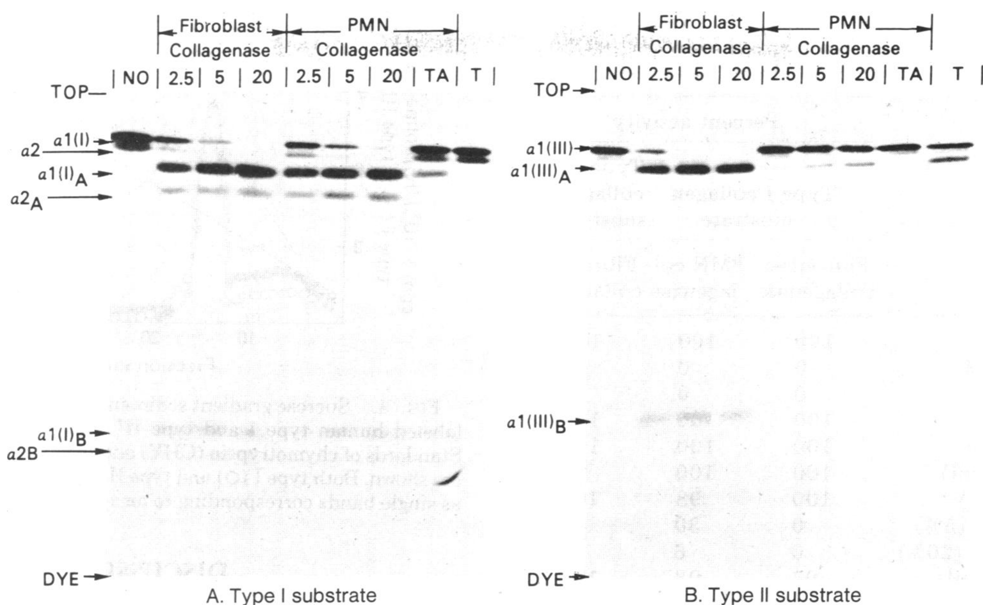

FIG. 2. Fluororadiograms of sodium dodecyl sulfate/acrylamide slab gel electrophoresis of human type III [^{3}H]collagen incubated with human fetal lung fibroblast (HFL-1) collagenase or human polymorphonuclear leukocyte (PMN) collagenase. (*A*) Type I [^{3}H]collagen (18 × 10^4 dpm) was incubated with: (NO = no enzyme), 2.5, 5, or 20 μl of HFL-1 collagenase (0.026 μg/μl); 2.5, 5, or 20 μl of concentrated PMN collagenase (0.195 μg/μl); TA = 1.2 μl (0.23 μg) of trypsin-activated PMN collagenase (trypsin 10 μg/ml, 20 min, 23°, followed by soybean trypsin inhibitor at 50 μg/ml); or T = 2 μg trypsin. Intact $\alpha 1$(I) and $\alpha 2$ chains and the collagenase digestion products $\alpha 1(\mathrm{I})_A$, $\alpha 2_A$, $\alpha 1(\mathrm{I})_B$, and $\alpha 2_B$ of the type I substrate are indicated. (*B*) Type III [^{3}H]collagen substrate (18 × 10^4 dpm) was incubated under identical conditions and enzymes as *A* and the resultant products were electrophoresed on the same acrylamide slab as *A*. Intact $\alpha 1$(III) chains and the collagenase digestion products $\alpha 1(\mathrm{III})_A$ and $\alpha 1(\mathrm{III})_B$ of the type III substrate are indicated. The faint band beneath the type III collagen $\alpha 1$(III) chains may be $\alpha 2$ chains from minor contamination of the type III substrate with type I collagen. Quantitative scans of this region demonstrated that the total radioactivity in the "$\alpha 2$" band was less than 2% of the total radioactivity in the $\alpha 1$(III) band. Rabbit pulmonary alveolar macrophage collagenase digested the human I and III substrates at the same rate (data not shown).

susceptible to trypsin, which cleaved it into two pieces, approximately 75% and 25% of the original molecule (Fig. 1*B*), as described by Miller *et al.* (22).

The differences between the human fibroblast and PMN collagenases in their attack of rabbit collagens I and III were also seen when human types I and III collagens were used as the substrates (Fig. 2*B*). When identical amounts of fibroblast collagenase were used against the human I and III substrates, both were attacked at the same rate (Fig. 3*A*) and appeared to have a similar spectrum of inhibition as with the type I substrate (Table 1). However, when identical amounts of PMN collagenase were used against the two substrates, the PMN enzyme attacked the type III collagen at 1/15 of the rate at which it attacked type I collagen (Figs. 2*B*, and 3*B*). Thus, under iden-

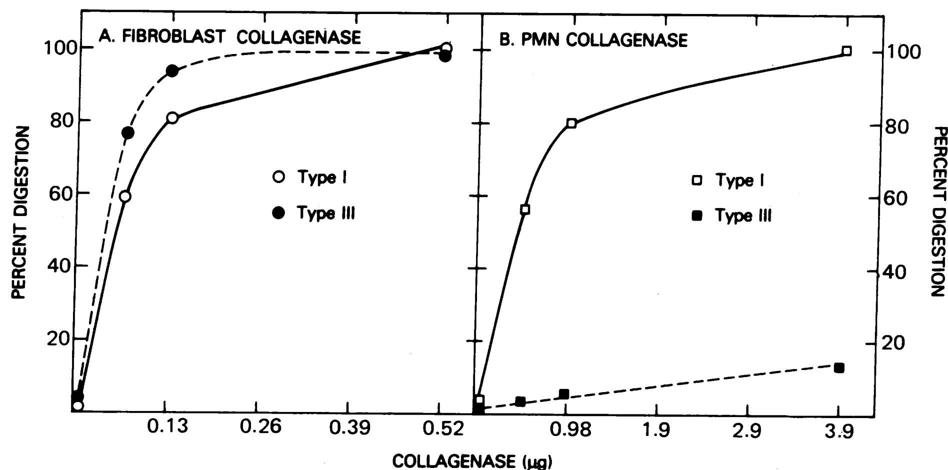

FIG. 3. Effects of increasing amounts of human fibroblast and human polymorphonuclear leukocyte collagenases on human type I and type III collagens. These data were derived from quantitative densitometric scans of the fluororadiograms of Fig. 2. Percent digestion was determined from the densitometric scans by quantitating the area under the α, α_A, and α_B bands of $\alpha 1$(I) (for digestion of type I collagen) and $\alpha 1$(III) (for digestion of type III collagen) using the formula: % digestion = 100 × [(area under α_A) + (area under α_B)]/[area under α]. (*A*) Digestion of human type I (○) and type III (●) collagen by human fibroblast collagenase. (*B*) Digestion of human type I (□) and type III (■) collagen by human PMN collagenase. With rate-limiting quantities of fibroblast collagenase (0.05–0.07 μg) approximately equal amounts of type I and type III collagens were degraded; with rate-limiting amounts of PMN collagenase (0.35–0.50 μg), 15 times more type I collagen was degraded than type III collagen.

Table 1. Effect of protease inhibitors on the cleavage of types I and III human collagen by fibroblast and polymorphonuclear leukocyte collagenase*

	Percent activity		
	Type I collagen substrate		Type III collagen substrate
Addition†	Fibroblast collagenase	PMN collagenase	Fibroblast collagenase
Enzyme only	100	100	100
+ EDTA (10 mM)	0	0	0
+ DTT (10 mM)	0	0	0
+ PMSF (2 mM)	100	100	100
+ NEM (10 mM)	100	100	100
+ BT-KI (10 U/ml)	100	100	98
+ BSA (8 mg/ml)	100	98	100
+ Human serum (5%)	0	30	0
(20%)	0	6	0
+ α1-AT (1 mg/ml)	97	98	100

* Reaction mixtures of fibroblast or PMN collagenase with human [^{3}H]proline-labeled type I or type III collagen substrate were incubated and electrophoresed as described in *Materials and Methods*. Fluororadiograms were prepared and integration of densitometric scans of the substrate was used to calculate the remaining percent activity after addition of potential collagenase inhibitors. The quantity of fibroblast collagenase used was sufficient to digest 90% of type I and type III collagen; this value was taken as 100% activity. The quantity of PMN collagenase used was sufficient to digest 90% of type I collagen; this value was taken as 100% activity. Because PMN collagenase digested so little of the type III substrate, quantitative studies with protease inhibitors could not be carried out. The rabbit pulmonary alveolar macrophage enzyme demonstrated the same spectrum of activity as the fibroblast enzyme with these inhibitors (data not shown).

† Abbreviations: EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; NEM, *N*-ethylmaleimide; BT-KI, bovine trypsin-kallikrein inhibitor (Aprotinin, Sigma); BSA, bovine serum albumin; α1-AT, human α1-antitrypsin (Worthington).

tical conditions in which the collagen substrate is in excess, equal amounts of PMN collagenase will cleave at least 15 times more type I collagen than type III collagen.

The relative amount of digestion of type I and type III collagen by PMN collagenase may be even greater than the 15 to 1 ratio suggested by the data in Fig. 3*B*. In the reaction mixtures demonstrating the incubation of PMN collagenase with type III substrate (Fig. 2*B*, slots 5–7), there were small amounts of collagen fragments in the α2 region, suggesting some of the $\alpha 1_A$ fragment could have been contributed by the action of PMN collagenase on small amounts of type I collagen contaminating the type III substrate. It is also possible that the small amount of type III digested by PMN culture media was not due to the same enzyme that attacked the type I substrate, but rather by another collagenase or by a "trypsin-like" enzyme (22). However, the latter is unlikely, since known trypsin inhibitors such as phenylmethylsulfonylfluoride and bovine trypsin–kallikrein inhibitor did not affect the small amount of type III digestion by PMN collagenase (data not shown).

In the evaluation of these studies, it is important to note that the type I and type III substrates were both in the collagen form rather than in aggregates, fibrils, or denatured. Both substrates were resistant to pepsin, neither would precipitate when centrifuged at 100,000 × *g* for 2 hr at 24°, and both sedimented at approximately 3.2 S (Fig. 4), corresponding to values previously reported for soluble collagen (23, 24).

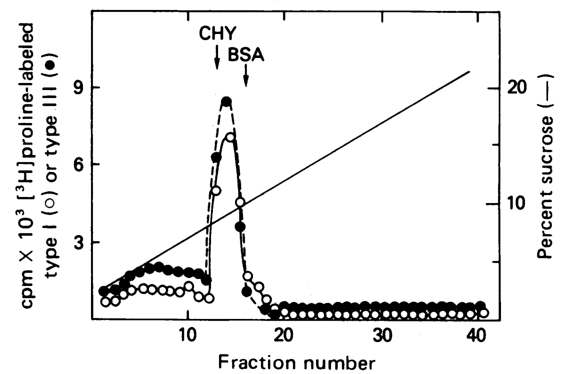

FIG. 4. Sucrose gradient sedimentation analysis of [^{3}H]proline-labeled human type I and type III collagens used as substrates. Standards of chymotrypsin (CHY) and bovine serum albumin (BSA) are shown. Both type I (○) and type III (●) [^{3}H]collagens sedimented as single bands corresponding to an $s_{20,w}$ of approximately 3.2 S.

DISCUSSION

These studies suggest that, under the conditions used, fibroblast and alveolar macrophage collagenases destroy type I or type III collagen indiscriminately, but polymorphonuclear leukocyte collagenase specifically cleaves type I collagen more rapidly than type III collagen. The experiments were designed such that it was possible to compare each collagenase against itself in its action against collagen types I and III; however, because the PMN and fibroblast enzymes were not pure, it is not possible to compare the relative rates of attack of the two different enzymes against a single collagen type.

It is important to note that the finding of an apparent specificity of PMN collagenase for type I collagen in comparison to type III collagen was not due to differences in the physical state of the collagen substrates used in the assays: (*i*) both the type I and the type III collagens were in a native, triple-helical form, as demonstrated by resistance to attack by pepsin and by sucrose gradient sedimentation analysis; and (*ii*) both collagen substrates migrated on reducing sodium dodecyl sulfate gels as α chains, and thus neither contained lysine-derived covalent crosslinks. In addition, even though the assay used soluble collagen substrates, estimates of the specificities of fibroblast and PMN collagenases for type I and type III collagens were made only when the quantity of enzyme was rate limiting (Fig. 3). Thus, the greater specificity of PMN collagenase for type I than for type III collagen appears to be an enzyme specificity rather than a result of a difference in the substrates. This is a very different situation from the relatively slow attack of type II collagen (compared to types I and III) by several collagenases, because this "specificity" is considered to be due to the difference in the substrates rather than the enzymes (12, 13, 25–29).

There was no evidence that the PMN medium contained a latent collagenase that would attack type III collagen (17), because concentrated PMN medium incubated with trypsin (followed by inhibition of trypsin with soybean trypsin inhibitor) digested type III collagen no better than unactivated medium (Figs. 1 and 2). Likewise, there was no evidence that the PMN collagenase preparation contained an inhibitor to a collagenase that attacks type III collagen, because a mixture of fibroblast and PMN collagenases cleaved the type III substrate at a rate equivalent to that due to the fibroblast collagenase alone. Finally, a mixture of types I and III collagen was completely digested by fibroblast collagenase, but only the type I component of the mixture was digested by PMN collagenase,

indicating that the type III substrate did not contain an inhibitor of collagenase (data not shown).

Even though there was a striking difference in the specificities of PMN collagenase and fibroblast collagenase, the two enzymes responded to a spectrum of inhibitors in a similar fashion (Table 1). The only exception was that PMN collagenase was more resistant to serum inhibition, as has been observed by others (11, 30). None of the collagenases tested was inhibited by α1-antitrypsin.

The data presented suggest that control of the location and quantity of collagen types I and III in different organs may be critically dependent on the ability of different cells to break down specific collagen types at different rates. The collagenase of the polymorphonuclear leukocyte, the first cell brought to an inflamed area, markedly favors degradation of type I collagen. This process may go unchecked, because PMN collagenase is relatively resistant to inhibition by serum inhibitors. At later stages of inflammation, this specificity is probably lost, because tissue (fibroblast) and/or macrophage collagenases attack type I and type III collagens at the same rate. In opposition to the specific attack of type I collagen by PMN collagenase, there may be a release of (as yet undescribed) trypsin-like enzymes that favor the destruction of type III collagen during some phase of inflammation.

These findings suggest that the changes in the relative proportions of collagen types I and III during inflammation and healing (type III early, type I late) (8–10) may be controlled, in part, by the collagenases present in the tissues. Thus, proteolysis of collagen may have a significant role in the control of the composition (and hence form and function) of the extracellular connective tissue.

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