HETEROGENEITY AMONG HUMAN COLLAGENASES
DEMONSTRATED BY MONOCLONAL ANTIBODY THAT
SELECTIVELY RECOGNIZES AND INHIBITS
HUMAN NEUTROPHIL COLLAGENASE

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The interstitial collagenases are a group of metalloproteinases that are capable
of degrading the interstitial collagens (types I, II, and III). Enzymes of this class
have been isolated from skin fibroblasts (1), synovial cells (2), macrophages (3),
and neutrophils (4). Despite the heterogeneity among the cells' origins, these
enzymes cleave native collagen molecules at the same amino acid residues. The
best-characterized of these enzymes are those derived from skin fibroblasts and
synovial cells. Both of these collagenases are released from the cell in a latent,
proenzyme form and can be converted to their active species by organic mercurial
compounds or by limited proteolysis with trypsin (as well as other proteinases)
(1, 5). These enzymes are more active against type III than type I collagen.
Extensive analysis of these two collagenases have indicated that they are nearly
identical.

Human neutrophil collagenase (HNC)1 is not nearly as well-characterized even
though it was one of the first to be described. This is in part due to the difficulty
of purification and to the relative instability of the enzyme. Nevertheless, there
are several reports suggesting that this collagenase may be quite different from
those from skin and synovial cells. HNC has been reported to be slightly larger
by molecular weight estimates (6), to degrade type I collagen more rapidly than
type III (7), and to be resistant to inactivation by α2-macroglobin (8). The
immunological data comparing HNC with other collagenases, however, have
been controversial. Bauer et al. (9) found synovial, gingival, and granulocyte
collagenase to cross-react identically with human skin collagenase in an immu-
noassay using antibody to skin collagenase. By contrast, Woolley et al. (10)
showed no reaction between granulocyte collagenase and antibody to rheumatoid
synovial collagenase. Previous reports of polyclonal antibodies against neutrophil

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1Abbreviations used in this paper: APMA, aminophenyl mercuric acetate; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HNC, human neutrophil collagenase; MEM, minimal essential medium; MNL, mononuclear leukocytes; NaSCN, sodium thiocyanate; PMA, phorbol myristate acetate; PMNL, polymorphonuclear leukocytes.

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collagenase have not addressed whether the neutrophil enzyme is distinct from the skin and synovial collagenases. In this report, we have shown human neutrophil collagenase to be immunologically distinct from human synovial and skin collagenase using a rigidly defined monoclonal antibody. The recognition that neutrophil collagenase is distinct from other collagenases underlines the importance of precise identification of the source of the collagenase present in inflammatory diseases such as rheumatoid arthritis (11) and idiopathic pulmonary fibrosis (11) in our understanding of the pathogenesis of these diseases.

Materials and Methods

**Leukocyte Isolation and Stimulation.** Leukocytes were isolated from whole blood by dextran sedimentation, washed three times with phosphate-buffered saline (0.02 M phosphate, 0.15 M NaCl, pH 7.4), followed by hypotonic lysis of contaminating erythrocytes according to the method of Boyum (13). The leukocyte fraction was resuspended at a concentration of 10^7 cells/ml in Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY) containing Ca^{++} and Mg^{++} and phorbol myristate acetate (PMA) (50 ng/ml; Sigma Chemical Co., St. Louis, MO) to induce granule release (14). After a 15 min incubation at 37°C, the cells were removed by centrifugation. Phenylmethylsulfonyl fluoride was added to a final concentration of 0.001 M and the supernatants were frozen. In some experiments, the mononuclear cells were separated from the granulocytes by centrifugation on Ficoll-Hypaque (13). After Ficoll-Hypaque centrifugation, the granulocytes were further purified by dextran sedimentation and washed with phosphate-buffered saline and the erythrocytes were removed by hypotonic lysis. Granule release was induced as described above.

**Preparation of the Immunogen.** After dialysis against 0.01 M Tris-HCl, 0.005 M CaCl_2, 0.02% NaN_3 at pH 8.3, the leukocyte supernatants were applied to a column of DEAE Affi-Gel Blue. The enzyme was eluted with a gradient of 0–0.7 M NaCl in 0.01 M Tris-HCl, 0.005 M CaCl_2, pH 8.3. The peak of collagenolytic activity was pooled, dialyzed against 0.05 M Tris-HCl, 0.5 M NaCl, 0.005 M CaCl_2, 0.02% NaN_3, pH 7.6, concentrated by pressure dialysis, and chromatographed on an ACA 34 gel filtration column. Those fractions showing collagenase activity were pooled, dialyzed against phosphate-buffered saline, concentrated by pressure dialysis, and frozen for use as an immunogen and an antigen in the enzyme-linked immunosorbent assay (ELISA).

**Hybridoma Selection and Screening.** Mice were immunized intradermally with 20 μg of partially purified collagenase from the gel filtration column in complete Freund's adjuvant. 30 d after the initial immunization, they received an intravenous boost with the antigen in saline. 3 d after the secondary immunization, the spleen cells were fused with the mouse myeloma line SP 2/0 using polyethylene glycol (PEG; Aldrich Chemical Co., Milwaukee, WI) as previously described (15).

Hybrids were assayed for antibody activity using an ELISA against the immunogen. For this assay, the culture medium was supplemented with serum that had been acid-treated to inactivate α2-macroglobulin. Cultures were subcloned two additional times in methylcellulose.

**Immunological Analysis.** Antibodies were assayed in an ELISA (16, 17). The wells of a microtiter plate were coated with 100 μl of partially purified neutrophil collagenase (5 μg/ml) in 0.05 M Tris, 0.15 M NaCl, pH 7.6 at 4°C overnight. Excess protein binding sites were blocked with the addition of 50 μl of 1% bovine serum albumin (BSA) with an additional incubation of 1 h at room temperature followed by five washes with ELISA buffer (0.05 M Tris, 0.15 M NaCl, 0.05% Tween 20). 100 μl of culture medium or ascites fluid diluted in ELISA buffer containing 0.1% BSA was incubated on the plates for 2 h at room temperature and the plates were again washed five times with ELISA buffer. A peroxidase-conjugated goat anti-mouse immunoglobulin (100 μl; Cappel Laboratories, Cochranville, PA) at a 1:1,500 dilution in 0.1% BSA in ELISA buffer was added and
incubated for 1 h at room temperature. After the wells were again washed five times, 100 μl of substrate, o-phenylenediamine (Sigma Chemicals Co.), was added. The substrate was prepared by dissolving 40 mg of o-phenylenediamine in buffer freshly prepared by mixing 24.3 ml of 0.1 M citric acid and 25.7 ml of 0.2 M Na₂HPO₄ and 50 ml distilled water, pH 5.0. 40 μl of 30% hydrogen peroxide was added. The reaction product was measured at 450 nm on a Microelisa Autoreader MR 580 (Dynatech Laboratories, Inc., Alexandria, VA).

For competitive inhibition studies, 150 μl of a 1:10,000 dilution of ascites fluid was preincubated at 37°C for 1 h with an equal volume of varying amounts of collagenase before incubation on the ELISA plate. The human neutrophil collagenase used as a solid phase and competitive antigen in these studies was isolated from the monoclonal antibody affinity column and had a concentration of 312 ng/ml.

**Immunoaffinity Column.** Hybridoma cultures were grown in serum-free Dulbecco's minimum essential medium (MEM) supplemented with nonessential amino acids, penicillin, streptomycin, and insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml) (ITS-Premix; Collaborative Research, Inc., Waltham, MA). The culture supernatants were pooled, dialyzed against 0.5 M NaCl, 0.1 M NaHCO₃, pH 8.2, and concentrated 10-fold by pressure dialysis. The serum-free concentrate was then covalently linked to cyanogen bromide-activated Sepharose (Bio-Rad Laboratories, Rockville Centre, NY). Crude neutrophil supernatants were dialyzed into 0.05 M Tris, 0.15 M NaCl, 0.005 M CaCl₂, 0.02% NaN₃, pH 7.6 and chromatographed on the antibody affinity column. The columns were washed with 20 vol of buffer and eluted with 6-8 vol of 3.5 M sodium thiocyanate (NaSCN). After dialysis of the eluate against the starting buffer, all fractions were assayed for collagenase activity using soluble type I collagen as substrate.

**Collagenase Assay.** Type I collagen was extracted from fetal calf skin and purified by the method of Glimcher et al. (18). The purified collagen was radiolabeled using [¹⁴C]acetic anhydride (19). Collagenolytic activity was measured by either determining the release of radioactivity from reconstituted collagen fibrils (20) at 37°C or by the incubation of the enzyme with soluble collagen at 25°C. In the soluble assays, 0.05 M arginine was included to prevent fibril formation and the degradation of collagen was assessed by separation of the reaction products on 7.5% polyacrylamide gels. Assays were run in the presence and absence of 1 mM aminophenyl mercuric acetate (APMA) to determine the amount of latent enzyme present (21). In experiments where the inhibitory activity of the monoclonal antibody was examined, the hybridoma culture supernatants were preincubated with neutrophil supernatants for 1 h at room temperature before the assay for collagenolytic activity.

Sodium Dodecyl Sulfate Gels. Electrophoresis was performed according to the method of Laemmli (22) using a Bio-Rad slab gel apparatus. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 45% methanol, 7% acetic acid overnight at room temperature and destained in 10% methanol, 7% acetic acid.

**Protein Determination.** Protein concentration was assayed using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories).

**Human Mesenchymal Collagenases.** Purified human skin collagenase was provided by Dr. George Stricklin and Dr. Arthur Eisen, Washington University School of Medicine, St. Louis, MO. Purified human rheumatoid synovial collagenase was obtained from Carol Vater and Dr. E. D. Harris, Jr., Dartmouth Medical School, Hanover, NH.

**Results**

**Collagenolytic Activity in Enriched Mononuclear (MNL) and Polymorphonuclear (PMNL) Leukocyte Fractions.** Previous studies have suggested that the MNL fraction is a poor source of collagenase and that this collagenolytic activity is not stored but rather requires new protein synthesis. Thus, the contribution of MNL to the collagenolytic activity secreted during this short incubation period should be minimal. However, to ascertain that the MNL in the crude leukocyte prepa-
rations routinely used were not the source of the enzyme generated, the MNL and PMNL fractions were separated by isopyknic centrifugation on Ficoll-Hypaque. Both cell populations were resuspended at $10^7$ cells/ml and stimulated with 50 ng/ml PMA for 15 min at 37°C. The supernatants from these cultures were tested for collagenolytic activity by incubating equal amounts of culture supernatant with reconstituted collagen fibril for 18 h at 37°C in the presence and absence of APMA. No collagenolytic activity was noted in MNL culture supernatants even in the presence of APMA (Table I). In contrast, culture supernatants from PMA-stimulated PMNL showed significant collagenolytic activity in the presence but not in the absence of APMA. These results indicate that the collagenolytic activity generated from the leukocyte fraction after stimulation by PMA was derived from PMNL and was present in latent form. Thus, the immunogen and screening antigen were derived from the PMNL.

Characterization of Monoclonal Antibody with Inhibitory Activity Against HNC. When colonies of hybrid cells were assayed by the ELISA, six of the wells reacted with the immunogen and were screened further for their ability to inhibit the collagenolytic activity of crude neutrophil supernatant against a collagen fibril substrate. Medium from only one showed significant and reproducible inhibition of collagenase activity. This clone was then recloned twice. The antibody secreted by this clone was used for all further experiments.

Although the inhibition of enzyme activity was suggestive evidence that an antibody to neutrophil collagenase had been isolated, the possibility existed that the antibody was interfering with the activation of latent collagenase instead of directly inhibiting the enzyme's action against the collagen substrate. To differentiate between these modes of inhibition, the culture medium containing the antibody was added either to neutrophil supernatant containing a latent enzyme or to the same sample with APMA present to activate the enzyme. The enzyme without APMA resulted in minimal lysis as compared with the sample containing enzyme plus APMA, as would be expected from a latent enzyme (Table II). Antibody added after APMA activation of the latent enzyme inhibited enzyme activity equally as well as antibody added before APMA activation. These results indicate that the antibody inhibition of collagenase activity was due to its interaction with the enzyme directly and not to any effect on the activation process.

The above studies suggested that the neutralizing monoclonal antibody was specific for activated neutrophil collagenase, but did not show if the antibody would bind latent collagenase as well. Therefore, the antibody was linked to

| Table 1 |
| Collagenase Activity of Mononuclear vs. Granulocyte Fraction |
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| **Fraction** | **Percent total cells** | **Percent collagen lysis** |
| | | −APMA | +APMA |
| Mononuclear | 100 | 0 | 0 |
| Granulocyte | 95 | 0.5 | 69.9 |

* Each sample contained 100 µg of collagen. Percent lysis was calculated as [counts released/counts released by bacterial collagenase] × 100 after subtraction from all values of the counts released by 10 µg of trypsin.
Table II
Antibody Inhibition of Human Neutrophil Collagenase: Inhibition of Enzyme Activity vs. Inhibition of Activation of Latent Collagenase

| Sample | Preactivation antibody addition | APMA | Postactivation antibody addition | Percent collagen lysis* |
|--------|--------------------------------|------|---------------------------------|------------------------|
| 1      | -                              | -    | -                               | 13.0                   |
| 2      | -                              | +    | -                               | 38.5                   |
| 3      | +                              | +    | -                               | 15.3                   |
| 4      | -                              | +    | +                               | 16.2                   |

* Collagen fibrils (100 μg/sample) were incubated with the enzyme overnight at 37°C.

Figure 1. Type I collagen after digestion with neutrophil collagenase with (+) or without (−) APMA to effect activation. (a) Crude enzyme, (b) crude enzyme after passage over the antibody column, and (c) subsequent elution of the enzyme from antibody column with NaSCN.

cyanogen bromide-activated Sepharose, and crude neutrophil supernatants containing latent collagenase were chromatographed. Analysis of these supernatants for enzyme activity by incubation with substrate in the absence and presence of APMA indicated that the enzyme applied to the affinity columns was predominantly in the latent form (Fig. 1 a). When applied to the column, the majority of the enzyme activity was bound. The small amount of activity seen in the material appearing in the initial wash-through (Fig. 1 b) appeared to be due to the overloading of the column because, upon rechromatography, all of this material bound to the column (data not shown). After elution with 3.5 M NaSCN, the collagenase was active even in the absence of APMA (Fig. 1 c). Taken together with the inhibition studies, these results indicate that the antibody recognized both latent and active forms of neutrophil collagenase.

Cross-reactivity with Human Mesenchymal Collagenases. Although the immunoabsorbent column suggested that the monoclonal antibody was capable of recognizing both latent and active collagenase, its value as a probe to differentiate neutrophil collagenase from other human collagenases remained to be determined. The cross-reactivity of the antibody was assayed by two methods (a) screening for inhibition of enzyme activity, and (b) using the ELISA to test for
the ability of other human collagenases to competitively inhibit the antibody's reaction with neutrophil collagenase.

To test for inhibition of enzyme activity against other human collagenases, culture medium from hybrid cells grown in acid-treated serum was preincubated with human skin collagenase, human rheumatoid synovial collagenase, or crude human neutrophil collagenase before incubation in a collagen fibril assay. The collagenase activity of each preparation had been previously calibrated and standardized to result in 20–50% of total lysis. As shown in Table III, only neutrophil collagenase was substantially inhibited in the presence of the antibody. However, the small amount of inhibition of human rheumatoid synovial collagenase made it necessary to define the immunological cross-reactivity. Using each of the other collagenases in a competitive inhibition ELISA against affinity-purified neutrophil collagenase as the solid-phase antigen, no cross-reactivity of the monoclonal antibody was seen with human skin or rheumatoid synovial collagenase even with quantities of the enzymes that were 10-fold greater than

**Table III**

Inhibition of Various Human Collagenases by Monoclonal Antibody

| Enzyme                          | Enzyme activity after preincubation with antibody in acid-treated serum medium (percent of control)* |
|---------------------------------|--------------------------------------------------------------------------------------------------|
| Human skin collagenase         | 101                                                                                               |
| Human rheumatoid synovial collagenase | 83                                                                                             |
| Human neutrophil collagenase   | 47                                                                                               |

* Controls were enzyme that had been preincubated with medium containing acid-treated serum alone. Human skin collagenase lysed 22.1 μg collagen total, human rheumatoid synovial collagenase lysed 25.3 μg collagen total, and human neutrophil collagenase lysed 45.6 μg collagen total.

![Figure 2](image-url)

**Figure 2.** Competitive inhibition of monoclonal antibody 11b.1 with human neutrophil collagenase (HNC), skin collagenase (HSKC), or rheumatoid synovial collagenase (HRSC) against HNC as the solid-phase antigen.
that of affinity-purified neutrophil collagenase (Fig. 2). By contrast, 80 ng/ml of neutrophil collagenase gave 100% inhibition in the ELISA. Therefore, neutrophil collagenase is an immunologically distinctive enzyme from other human collagenases.

Discussion

The interstitial collagenase of the neutrophil has been implicated in several pathologic states where collagenase activity in body fluids has been found in association with large numbers of neutrophils (12, 23). Indirect evidence for a pathogenic role of neutrophil collagenase stems from the work of Harris et al. (11) who identified collagenase in rheumatoid synovial fluid that did not resemble the synovial enzyme with respect to molecular weight and its resistance to inactivation by α2-macroglobulin. However, the lack of specific probes has hampered efforts to assess the role of this enzyme in both physiologic and pathologic processes.

The scarcity of reports using antibody to neutrophil collagenase may be due in large part to the difficulty of preparing the pure antigen. Freshly isolated neutrophils must be used since, unlike human skin or synovial cells, these cells do not continuously synthesize this enzyme but store it in a preformed granule. During purification, substantial activity is lost and the specific activity of the isolated enzyme is low in comparison with human skin or rheumatoid synovial collagenase (24).

Two groups have reported the development of polyclonal antibodies to neutrophil collagenase. Ohlsson and Olsson (25) described an antibody to neutrophil collagenase. However, their antibody reacts with a serine proteinase that localizes to the azurophil granule. This conflicts with the data that neutrophil collagenase is a metalloproteinase located in the specific granule (6). More recently, Christner and co-workers (26) have described the isolation of a polyclonal antibody to neutrophil collagenase. The cross-reactivity of their antibody to other human collagenases was not examined. Our antibody, however, unequivocally recognized neutrophil collagenase and shows no cross-reaction with human skin or human rheumatoid synovial collagenase. In view of the ability of our monoclonal antibody to distinguish clearly between neutrophil and mesenchymal cell collagenases, the previous reports of the larger molecular weight of neutrophil collagenase (6), and the resistance of the latter to inactivation by α2-macroglobulin (27), it is most likely that neutrophil collagenase represents a gene product that is distinct from the other mesenchymal collagenases. Its mechanisms of activation and inhibition as well as those secretagogues that promote its release may well be different from those previously described for mesenchymal collagenases.

Summary

The heterogeneity of human collagenases has been examined using a monoclonal antibody to neutrophil collagenase. This antibody inhibited collagenase activity and, when covalently coupled to Sepharose, bound both latent and active enzyme. Although human neutrophil collagenase was inhibited by the antibody,
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the activity of human skin and rheumatoid synovial collagenase was not signi-
ificantly diminished in the presence of the antibody. Competitive inhibition studies
also differentiated between these collagenases. Only human neutrophil collagen-
ase effectively blocked the antibody in a competitive enzyme-linked immuno-
sorbent assay while skin and rheumatoid synovial collagenase again failed to
interact with the antibody. The unequivocal recognition of neutrophil collagen-
ase as an immunologically distinct entity from other collagenases supports the
hypothesis that neutrophil collagenase is a separate gene product from fibroblast
or synovial collagenase.

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