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Solid-Phase Radioimmunoassay for Human Neutrophil Elastase: A Sensitive Method for Determining Secreted and Cell-Associated Enzyme[1]

THADDEUS L. DUNN, WARREN D. BLACKBURN, WILLIAM J. KOOPMAN, AND LOUIS W. HECK

Departments of Medicine and Microbiology, University of Alabama in Birmingham School of Medicine, Birmingham, Alabama 35294

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A solid-phase radioimmunoassay for measuring neutrophil elastase in the range 0.08–4 ng/ml has been developed. A monospecific, precipitating antibody capable of inhibiting elastinolysis was produced by repeated immunizations of a goat. The IgG fraction and affinity-purified antibodies of this serum were then obtained and used to develop this radioimmunoassay. There was no cross-reactivity in binding of the radiolabeled antisera with lactoferrin, cathepsin G, or serine proteinases with amino-terminal amino acid sequence homology. Although serum influences the measurement of catalytically active neutrophil elastase when compared to diisopropylfluorophosphate-treated neutrophil elastase, antigenic elastase may still be measured in body fluids. Furthermore, this assay is more sensitive than commercially available substrates used for quantitating neutrophil elastase by functional activity. We have found this quantitative assay extremely useful in balance studies to measure secreted and cell-associated elastase and in screening of biological fluids for the presence of the enzyme.

The extracellular release of human neutrophil elastase (HNE)[2] into the inflammatory microenvironment may be associated with tissue injury or destruction. Efforts to precisely quantitate the extracellular release of this proteinase from neutrophils have been hampered not only by limitations in the sensitivity and specificity of previous studies using enzymatic (1,2) and immunologic assays (1) but also by the presence of molecules in body fluids which may influence measurement of this activity (3). Furthermore, neutrophil elastase activity has been shown to be affected by substrate concentration, the presence of organic solvents, and variations in ionic strength, which thus complicates the quantitation of this enzyme (4). The only reported sensitive immunoassay for a neutrophil elastase has been for dog neutrophil elastase (5). In this report we describe the development of a sensitive and specific solid-phase radioimmunoassay (RIA) which has been found to be useful in the quantitation of secreted and intracellular forms of neutrophil elastase and in screening many samples of body fluids for the presence of the enzyme.

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[2] Abbreviations: HNE, human neutrophil elastase; HNCG, human neutrophil cathepsin G; PMN, polymorphonuclear leucocyte (neutrophil); DFP, diisopropylfluorophosphate; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; Suc-$(Ala)_3$-pNa, *N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanalide; MeO-Suc-Ala-Ala-Pro-Val-pNa, methoxysuccinyl-L-alanyl-L-alanyl-L-proline-L-valine-*p*-nitroanalide; MeO-Suc-Ala-Ala-Pro-Val-AMC, methoxysuccinyl-L-alanyl-L-alanyl-L-proline-L-valine-methylcoumarin amide; NP-40, Nonidet-P40; IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; RIA, radioimmunoassay.

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MATERIALS AND METHODS

Materials. Phosphate-buffered saline (PBS) without calcium and magnesium, and Hank's balanced salt solution (HBSS) were from GIBCO, Grand Island, New York; BSA (RIA grade), diisopropylfluorophosphate (DFP), lactoperoxidase, and zymosan were from Sigma Chemical Company, St. Louis, Missouri; Immulon II polystyrene wells were from Dynatech Laboratories, Alexandria, Virginia; MeO-Suc-Ala-Ala-Pro-Val-pNa, Suc-Ala-Ala-Ala-pNa, and urokinase were from Calbiochem-Behring, San Diego, California; and Meo-Suc-Ala-Ala-Pro-Val-AMC, and H-AMC were from Peninsula Laboratories, San Carlos, California. The sources of Sepharose 4B, 4-nitrophenol, insoluble elastin, elastase (porcine), chymotrypsin (bovine), trypsin (bovine), and ^{125}I-Na were from the sources stated in the accompanying paper (6).

Purification of HNE. HNE was purified by sequential sodium chloride extraction, Aprotonin-Sepharose affinity chromatography, *O*-(Carboxymethyl)-cellulose ion-exchange chromatography, and AcA_{44} gel filtration chromatography as described in the accompanying paper (6).

Isolation of other proteins. Cathepsin G was isolated from purified neutrophils by the procedure of Travis *et al.* (7). Lactoferrin from human breast milk and monospecific antibody to this protein were provided by Dr. Jiri Mestecky (UAB) as described (8). Plasminogen was isolated from normal human plasma and activated with urokinase as described (9). Factor D was a generous gift of Dr. Marilyn Niemann (UAB) (10). Bovine thrombin was prepared from topical thrombin as described (11).

Production of a polyclonal goat antibody specific for neutrophil elastase. A male goat, approximately 1 year old, was injected subcutaneously and intramuscularly with 25 μg of HNE which had previously been treated with DFP at a final concentration of 10^{-4} M. The inactivated enzyme was dialyzed extensively against phosphate-buffered saline and emulsified with an equal volume of Freund's complete adjuvant. Immunization was repeated every 2 weeks for a total of 12 weeks as described above except that the antigen was emulsified with Freund's incomplete adjuvant. Nonimmune serum was obtained from the same goat prior to immunization. Control sera and antisera were stored at −20°C with 0.1% sodium azide added as a preservative.

The IgG fraction of preimmune and immune sera was isolated by sequential ammonium sulfate precipitation and DEAE-cellulose (DE-52) chromatography (12). Affinity columns to isolate antibody were made by coupling purified HNE to Sepharose 4B at a concentration of 100 μg of protein/ml of the settled gel by the cyanogen bromide procedure (13). After the gel was washed to remove nonspecifically absorbed enzyme, the enzyme was then inactivated by incubation in DFP at a final concentration of 10^{-4} M for 1 h at room temperature. The excess DFP was removed by washing with borate saline, pH 8.2, and 1 ml of the goat antisera was applied to a 2-ml column of the HNE-Sepharose 4B in borate-buffered 0.5 M NaCl, pH 8.2, and washed until the absorbance at 280 nm was less than 0.02. The affinity purified antibodies were eluted with 0.1 M glycine-HCl, pH 2,8, and the pH of the eluate was adjusted to pH 8.0 by the addition of 2 M Tris-HCl. The eluate was dialyzed into PBS, and concentrated by vacuum dialysis, and protein content was determined by measuring the absorbance at 280 nm using $E^{1\%}_{1\,cm} = 13.5$ as the extinction coefficient. The protein content was adjusted to 1 mgm/ml with PBS and aliquots were stored frozen at −70°C.

Antibody specificity studies were performed using double immunodiffusion, electroblotting, inhibition of elastinolysis, and binding assays. Double immunodiffusion was performed in agar by a modification of the method described by Garvey *et al.* (14) in which the agar was suspended in 1.0 M NaCl containing 0.25% NP-40. Typically, 1–10 μg of purified proteins or lysates pretreated with

10^{-4} M DFP was placed in the peripheral wells and antisera in the center well. Immunoblotting experiments were performed after neutrophil lysates were separated using SDS–polyacrylamide gradient gel electrophoresis under reducing conditions. The preparation of the gel, activation of diazo paper, and transfer conditions were performed as recommended by the manufacturer. The IgG fractions of preimmune and immune goat sera were iodinated by the lactoperoxidase technique (see below) and immunoblotting was performed according to the method of Ehrlich *et al.* (15). The paper was then washed extensively to remove nonspecific iodinated antibody probes, dried, and subjected to autoradiography.

Radioiodination of protein. Affinity purified anti-HNE antibody was labeled with carrier-free ^{125}I-Na by using a modification of the lactoperoxidase method (16). Generally, 10-μg quantities of protein were labeled to an initial specific activity of 2.2×10^{-5} mCi/ng. Free iodine was separated from bound ^{125}I by Sephadex G-25 column chromatography. The ^{125}I-labeled anti-HNE was aliquoted and stored at −70°C for up to 1 month.

Method of radioimmunoassay. Variations in the dilutions of reagents, incubation time, and washing conditions were investigated and the optional radioimmunoassay conditions were found to consist of the following: DE-52 cellulose-purified IgG fraction goat anti-HNE (10 mg/ml), diluted at 1/1000 in PBS, was used to coat vinyl assay wells (125 μl) for 4 h at 25°C. The wells were then blocked with PBS–1% BSA (100 μl) for 1 h at 25°C to eliminate nonspecific binding and washed with PBS three times, and 100-μl samples were then added to each well and allowed to incubate 16 h at 25°C. Standard curves were generated with serial dilutions of the stock DFP-inactivated enzyme (500 μg/ml) in PBS–1% BSA. After three washings with PBS, affinity purified ^{125}I-labeled anti-HNE was added to each well (100,000 cpm/100 μl). The wells were incubated for 16 h at 25°C and washed three times with PBS, and each well was counted for 1 min in a gamma counter. ^{125}I-Anti-HNE (cpm bound) $\times 10^{-3}$ was plotted against protein concentration in nanograms per milliliter. Standard binding curves using other purified proteins instead of HNE were used as described above.

Zymosan preparation. Zymosan particles (50 mg) were suspended in 10 ml of normal saline and placed in a boiling water bath for 1 h. The preparation was then diluted with 50 ml of normal saline, centrifuged, decanted, and washed three additional times. The pellet was diluted by adding 100 ml of normal saline so that there were 5×10^7 particles/ml. For endocytosis experiments, 200 μl of suspended zymosan particles or normal saline was added to neutrophils.

Secretion experiments. Neutrophils were prepared from heparinized whole blood by dextran sedimentation and isopycnic centrifugation as described in the preceding paper (6). To measure total cell-associated elastase, 5×10^6 cells were suspended in HBSS-1% BSA in triplicate and centrifuged, and the pellet fraction was extracted in 1 ml of lysing buffer containing 1% NP-40, 1.0 M NaCl, 0.05 M Tris, pH 8.0, 10^{-4} M DFP with vigorous agitation for 30 min at 25°C. The mixture was centrifuged at 13,000 rpm for 5 min, the supernatant removed, and the pellet fraction reextracted in an identical manner. Approximately 86–92% of the total elastase was released in the first extraction and the remaining amount in the second extraction as determined by radioimmunoassay. To perform elastase balance studies, triplicate sets of cells were suspended in 1 ml of HBSS–1% BSA containing buffer or zymosan particles and incubated for 60 min at 37°C using an agitating water bath. DFP at a final concentration of 10^{-4} M was added to the supernatant fluid and the pellets were extracted using lysis buffer as described above.

Ten micrograms of purified human neutrophil cathepsin G, milk lactoferrin, plasminogen, plasmin, urokinase, factor D, porcine

elastase, bovine chymotrypsin, trypsin, and thrombin was incubated with DFP at a final concentration of 10^{-3} M for 30 min at 25°C, diluted in PBS–1% BSA, and assayed as described above.

The effect of adding active HNE to either freshly prepared human serum or PBS–1% BSA was investigated. Ten micrograms of active HNE was incubated in 1 ml of fresh serum or PBS–1% BSA for 30 min at 25°C, and then diluted in PBS–1% BSA and assayed.

Comparative sensitivity of the RIA and other assays used to measure enzymatic activity of neutrophil elastase. A number of functional assays have been described for measuring specific elastase activity and we wanted to compare the sensitivity of the RIA with some of these assays. Elastinolysis assays utilizing elastin–agarose (17) and [^{3}H]elastin (18) were performed. Zones of lysis of elastin–agarose were recorded after a 24-h incubation using an enlarged viewer. TCA-soluble [^{3}H]elastin digestion products were determined by counting a 100-μl aliquot of the supernatant fluid in 5 ml of Aquasol using a Beckman Model LS 8000 scintillation counter. Spectrophotometric assays using defined peptide substrates with chromogenic leaving groups (MeO-Suc-Ala–Ala–Pro–Val-pNA (2); Suc-Ala–Ala-pNA (19)) and fluorogenic leaving groups (Meo-Suc-Ala–Ala–Pro–Val-AMC (2)) were performed as described. End-point measurements were made after a 4-h incubation except for the elastin–agarose assay (see above). This time period was chosen because the enzyme activity was linear with time using these substrates with nanogram amounts of HNE. Absorbance measurements at 410 nm were made using a Hitachi 100-40 spectrophotometer. Fluorescence measurements were made using an Aminco-Bowman spectrophotofluorometer. The minimal detection limit was defined as that value which was 2 SD below the least measured value.

RESULTS AND DISCUSSION

Specificity of Goat Antibody to HNE

The immunological relationship of the purified HNE to a 1.0 M NaCl extract of purified neutrophil is seen in Fig. 1A. In this double-diffusion agar system, the two observed pre-

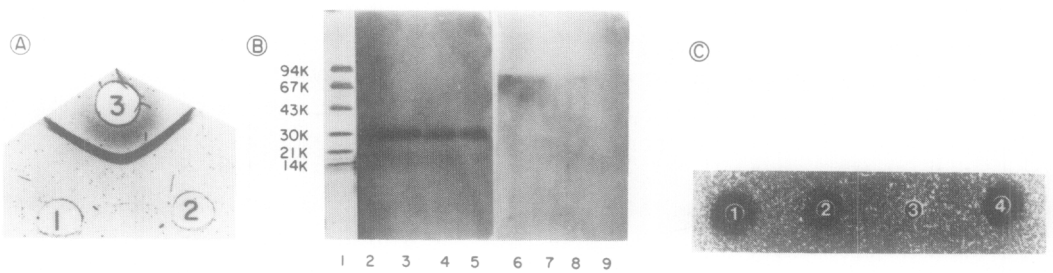

FIG. 1. The characterization of a goat antibody against HNE. (A) Coomassie blue-staining pattern of double immunodiffusion in agarose gel. The protein samples were loaded into agarose wells: well 1, 1 μg of purified HNE; well 2, 200 μg of neutrophil 1.0 M NaCl extract; well 3, goat antisera to HNE. (B) Autoradiography of SDS–polyacrylamide gradient gel electrophoresis run under reducing conditions. The protein samples loaded on this gel were: lane 1, molecular weight markers; lanes 2 and 6, 1 μg purified HNE; lanes 3 and 7, 4 and 8, and 5 and 9 were loaded with 300 μg of neutrophil extract from three different donors, respectively. After electrophoresis, the protein was transferred to diazotized cellulose paper; lanes 2–5 were incubated with iodinated goat antiserum to HNE and lanes 6–9 with undiluted goat preimmune ^{125}I-serum. (C) Inhibition of elastinolysis by affinity purified antibodies to HNE. Wells 1 and 2, 1 and 0.5 μg of purified HNE; well 3, 1 μg of HNE mixed with 10 μg of affinity purified antibodies against HNE; well 4, 1 μg of HNE mixed with 10 μg of affinity purified antibodies against lactoferrin. All incubations were for 7 days at 37°C.

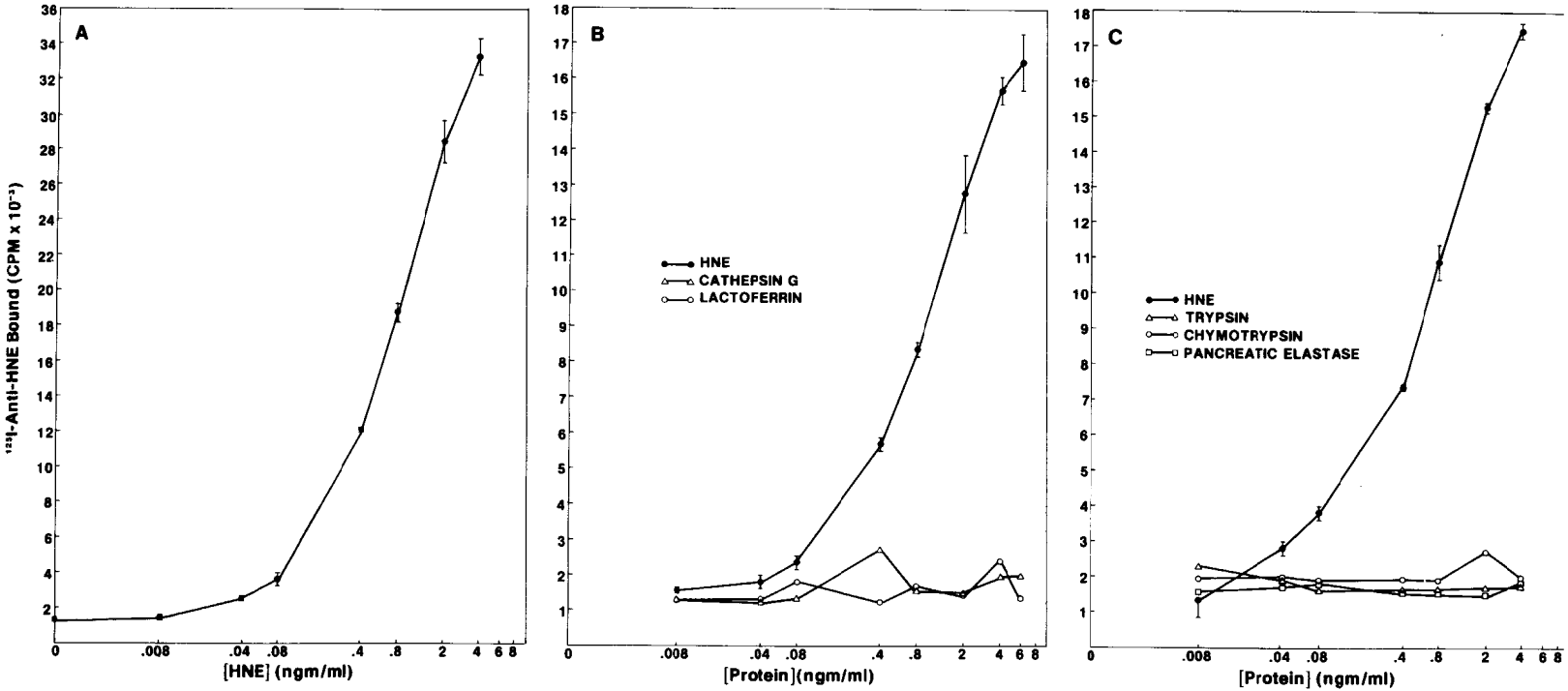

FIG. 2. Sensitivity and specificity of HNE radioimmunoassay. (A) The standard curve for the HNE radioimmunoassay. Details of the assay are described under Materials and Methods. Each point represents the average of triplicate measurements. Specificity of this assay using purified cathepsin G and lactoferrin (B) and pancreatic serine proteinases (C).

cipitin bands form a line of identity. To determine if there was a diversity of cell-associated antigenic forms of HNE, purified HNE and neutrophil lysates were subjected to SDS–polyacrylamide gradient gel electrophoresis under reducing and denaturing conditions. The separated proteins were then transferred to diazotized paper, exposed to the iodinated fraction of immune sera, washed, and subjected to autoradiography. The results are presented in Fig. 1B and demonstrate that the M_r of the purified HNE (lane 2) is very similar to the intracellular form(s) of this enzyme (lanes 3–5). In other immunoblotting experiments, no binding between HNCG or LF (1–10 ng) and our iodinated immune sera was observed (data not shown). Affinity purified antibodies to HNE, but not to lactoferrin, inhibited the proteolysis of insoluble elastin by HNE as shown in Fig. 1C.

Binding Sensitivity and Specificity of Solid-Phase Radioimmunoassay for HNE

The binding of ^{125}I-anti-HNE to known concentrations of DFP-inactivated HNE is depicted in Fig. 2A. Affinity purified radiolabeled anti-HNE bound in a linear fashion over a 50-fold concentration of HNE standards ranging from 80–4000 pg/ml. The minimal detection limit was 40 pg/ml. Variability of the assay was assessed by repeated testing of two purified elastase samples, (A) and (B), at different concentrations ($N = 4$; $\bar{x} \pm$ SD = 121 ± 17 pg/ml (A); 770 ± 62 pg/ml (B)).

The comparison of ^{125}I-anti-HNE binding to HNE with that of two purified neutrophil granule components, cathepsin G and lactoferrin, and isolated serine proteinases such as porcine pancreatic elastase, bovine chymotrypsin, human plasmin, thrombin, and factor D with greater than 40% sequence homology in the amino terminus, was performed. As can be seen in Figs. 2B and C there is no binding of ^{125}I-anti-HNE to these isolated granule components or other serine proteinases.

TABLE 1

ELASTASE RELEASE FROM HUMAN NEUTROPHILS AFTER EXPOSURE TO ZYMOSAN PARTICLES

Treatment	Elastase (ng)
1. Untreated 5×10^6 cells, cell associated[a]	6200 ± 250
2. Zymosan-treated 5×10^6 cells[b]	
Supernatant fluid	252 ± 40
Cell associated	5620 ± 240
3. Saline-treated 5×10^6 cells[b]	
Supernatant fluid	59 ± 4.7
Cell associated	6050 ± 340

[a] Cells extracted at time 0 and the total HNE measured.
[b] Cells were incubated for 1 hr, then supernatant and cell-associated HNE were measured.

Measurement of HNE Released from Isolated Neutrophil during Endocytosis

Secretion experiments were performed using 5×10^6 neutrophils suspended in HBSS–1% BSA incubated with and without zymosan. The incubation mixtures did not include cytochalasin B. Balance studies to measure elastase secreted into the media, residual cell-associated elastase after incubation, and the total amount in untreated cells were performed and the results from four experiments are shown in Table 1.

Neutrophils exposed to zymosan secreted 252 ± 40 ng elastase in the media whereas untreated cells secreted only 59 ± 4.7 ng. Cell-associated elastase after zymosan stimulation was considerably lower (5.62 ± 0.24 μg) than that for untreated cells (6.05 ± 0.34 μg). By using this assay, we could account for 95–98% of the total cell-associated elastase.

Influence of Serum on HNE Measurement

We hoped to be able to increase both the versatility of this assay and the scope of its application by measuring the elastase content

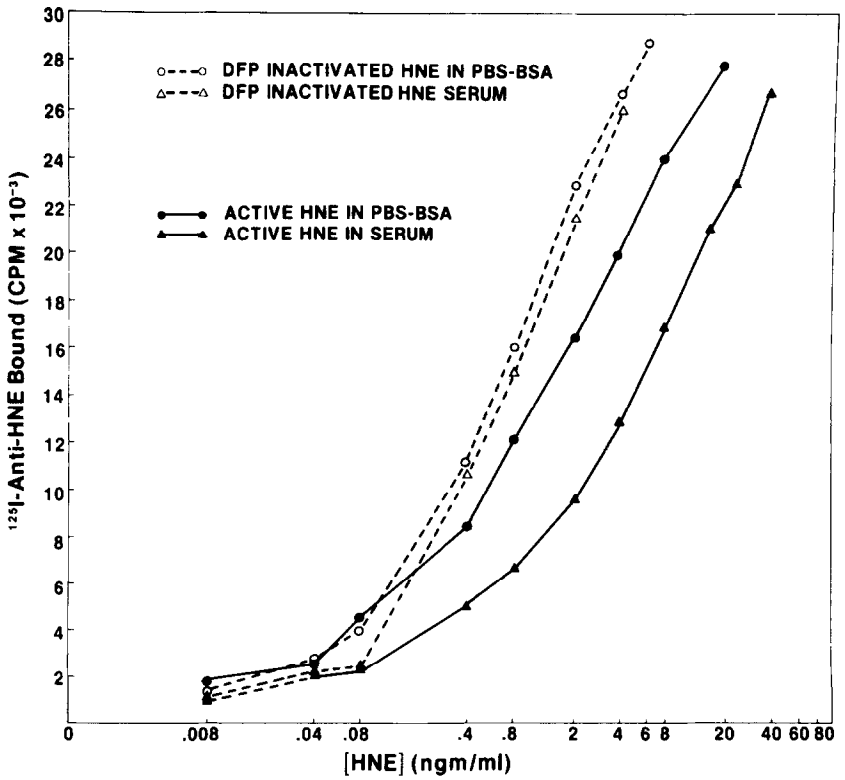

FIG. 3. The effect of serum on the quantitation of enzymatically active HNE and DFP-inactivated HNC. Details of the incubation mixture are described under Materials and Methods.

in complex biological mixtures. Serum contains many zymogens and inhibitors potentially capable of interacting with HNE which could potentially limit or inhibit antibody binding. As demonstrated in Fig. 3, the addition of inactive HNE to either PBS–1% BSA or human serum resulted in identical binding curves (Fig. 3). However, different binding

TABLE 2

SENSITIVITY OF VARIOUS ASSAYS FOR NEUTROPHIL ELASTASE

Assay	Detection limit (ng/10 μl diluted enzyme)	Detection limit (pmol)	Linear range of assay (ng)
1. Elastin–agarose	1000	33	1000–5000
2. [^{3}H]Elastin	125	4.1	250–1000
3. Suc-$(Ala)_3$-pNa	62.5	2.06	100–500
4. MeO-Suc-$(Ala)_2$–Pro–Val-pNa	5.0	0.12	7.5–125
5. MeO-Suc-$(Ala)_2$–Pro–Val-MCA	0.65	0.032	0.97–2.0
6. Radioimmunoassay	0.04	0.002	0.08–4

curves were obtained if active HNE was added to PBS–1% BSA or to serum (Fig. 3). We have been able to measure HNE using this assay (but not by functional assays) in inflammatory synovial fluid (L. Heck, unpublished observations).

Comparison in Sensitivity of the Radioimmunoassay with Functional Assays

Purified neutrophil elastase was diluted and incubated with defined elastase substrates under the described assay conditions except that the time of incubation was 4 h. In Table 2, the maximum sensitivity of each assay is reported and compared to our RIA. The results indicate that the RIA is more sensitive than functional assays using substrates which are commercially available.

In summary, the purpose of this study was to develop a sensitive and specific assay to quantitate HNE, an endopeptidase with broad substrate specificity thought to be important in inflammatory tissue injury. We prepared an antiserum to HNE in a goat that was monospecific with no cross-reactivity for cathepsin G, lactoferrin, or other serine proteinases with amino-terminal sequence homology. The assay described here is simple and sensitive, and is capable of measuring HNE at a concentration of 0.08–4 ng/ml. Balance studies to measure secreted and cell-associated HNE from neutrophils exposed to zymosan particles were performed and 96–98% of the total cellular elastase could be recovered. Extractions of pellets in buffers containing 1.0 M NaCl and 1% NP-40 did not interfere with HNE measurement. In a comparative study using commercially available substrates, our assay is more sensitive than those using elastinolysis or peptide substrates with chromogenic or fluorogenic leaving groups. This RIA should therefore be useful in quantitating HNE in bodily fluids and for studying quantitative aspects of HNE release in defined *in vitro* conditions.

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