A NEUTROPHIL-DEPENDENT PATHWAY FOR THE GENERATION OF A NEUTRAL PEPTIDE MEDIATOR

PARTIAL CHARACTERIZATION OF COMPONENTS AND CONTROL BY α-1-ANTITRYPSIN*

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Polymorphonuclear leukocytes have the capacity to supply proteolytic enzymes capable of generating kinin activity from a plasma protein substrate, kininogen, under diverse reaction conditions (1–3). Experiments, initially directed toward the mechanism of neutrophil-dependent kinin generation, have uncovered a different system which is controlled by α-1-antitrypsin. Incubation of crude neutrophil homogenates with heated human plasma generated a biologically active neutral peptide, distinct from bradykinin, derived from a plasma protein substrate chromatographically separable from kininogen. The neutrophil enzyme responsible for the formation of neutral peptide was a serine-dependent protease sensitive to inhibition by plant-derived trypsin inhibitors and α-1-antitrypsin.

Materials and Methods

Bradykinin triacetate (Sandoz Pharmaceutical, Ltd., Basel, Switzerland); hexadimethrine bromide (Polybrene) and diisopropyl fluorophosphate (DFP)[1] (Aldrich Chemical Co., Inc., Milwaukee, Wisc.); disodium ethylenediamine tetraacetate (EDTA) and tris hydroxymethyl aminoethane (Tris) (Fisher Scientific Co., Fair Lawn, N. J.); lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), pancreatic trypsin inhibitor (PI), ovomucoid trypsin inhibitor (OMTI), trypsin and chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.); Hanks' balanced salt solution (Microbiological Associates, Inc., Bethesda, Md.); Ficoll, Blue dextran 2000, aldolase, ovalbumin, quaternary aminoethyl (QAE) Sephadex A-25, Sephadex G-15, Sephadex G-150, Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.); human serum albumin (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); vitamin B12 (Eli Lilly and Co., Indianapolis, Ind.); sodium diatrizoate (Hypaque) (Winthrop Laboratories, N. Y.); and goat antihuman alpha$_1$ trypsin inhibitor (Miles Laboratory, Kankakee, Ill.) were obtained as noted. α-1-antitrypsin and α-2-macroglobulin (4) and

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[1] *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; LBTI, lima bean trypsin inhibitor; ng BK Eq, nanogram bradykinin equivalent; OMTI, ovomucoid trypsin inhibitor; PI, pancreatic trypsin inhibitor; QAE, quaternary aminoethyl; SBTI, soybean trypsin inhibitor; SRS-A, slow-reacting substance of anaphylaxis.

slow reacting substance of anaphylaxis (SRS-A) (5) were purified as previously described. The (null) α-1-antitrypsin-deficient plasma was obtained by Dr. Charles Reed (Univ. of Wisconsin) from the proband (6).

Column chromatography was carried out at 4°C. Sephadex G-15 and Sepharose 4B filtration were performed with a 1.5 cm × 90-cm glass column with a flow rate of 10.0 ml/h and a collection vol of 1.71 ml/tube. QAE Sephadex A-25 chromatography employed a 4.8 cm × 55-cm glass column with a flow rate of 50 ml/h and a collection vol of 12 ml/tube. Sephadex G-150 filtration was carried out in a 2.5 cm × 160-cm glass column pumped against gravity at a flow rate of 10 ml/h and a collection vol of 3 ml/tube. Protein was quantitated in column fractions by OD at 280 nm utilizing a Beckman DU-2 Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) or by Folin-Ciocalteu reaction. Chromatographic fractions were concentrated with positive pressure chambers and UM-10 membranes (Amicon Corp., Lexington, Mass.). Unless otherwise indicated centrifugation steps were carried out at 4°C in an International PR-2 or PR-6 centrifuge (International Equipment Co., Needham Heights, Mass.).

Smooth muscle contractile activity was routinely measured on an isolated, atropinized, mepyraminized guinea pig ileum obtained from 200-g Hartley guinea pigs; the ileum was suspended in Tyrode's solution and the contractile response standardized with synthetic bradykinin (7) and expressed as nanogram bradykinin equivalent (ng BK Eq). Kininlike activity was assessed on the estrous rat uterus suspended in DeJalon's solution (7). Reaction mixtures were directly bioassayed unless the procedure specifies concentration by lyophilization or flask evaporation.

Preparation of Crude Neutrophil Extract.—Human neutrophils and mononuclear leukocytes were isolated and purified from whole human blood by dextran sedimentation of red blood cells and fractionation of white blood cells by centrifugation on Ficoll-Hypaque cushions (8). Purified neutrophils were suspended in Hanks' solution (10^8 neutrophils/ml) and broken by repeated freezing with dry ice in acetone and thawing at room temperature. Broken cells were further fragmented for 3 min at 4°C by means of exposure to a probe sonicator (Ultrasonics Instruments International, Inc., Farmingdale, N. Y.). The cell debris was removed by centrifugation at 400 *g* for 20 min, and the supernate was retained as crude neutrophil extract.

Preparation of Heat-Inactivated Plasma.—Whole human blood, collected in EDTA (1.0 mg/ml) and hexadimethrine bromide (36.5 μg/ml), was centrifuged at 900 *g* for 20 min and the supernatant plasma harvested. Plasma was then heated for 2 h at 61°C to inactivate inhibitors, proteolytic enzymes, and plasma kallikrein, and centrifuged at 900 *g* for 5 min to remove precipitated materials. The resulting supernate was employed as heat-inactivated plasma. A variety of normal human donors were utilized interchangeably as sources for crude neutrophil extract and heat-inactivated plasma.

RESULTS

The time-course of generation of the ileal contracting principle was studied at 37°C in replicate reaction mixtures containing 100 μl of crude neutrophil extract derived from 10^7 neutrophils and 900 μl of heat-inactivated plasma. The reaction was stopped at varying time intervals by addition of 4.0 ml ice-cold absolute ethanol. After centrifugation for 10 min at 4°C, the supernate, which contained at least 90% of the contractile activity generated, was flask evaporated to dryness and resuspended in distilled water for bioassay. As depicted in Fig. 1, the generation of contractile activity proceeded rapidly, with the appearance by 10 min of 50% of the maximal activity demonstrable at 2 h. The pH of the standard reaction mixture of undiluted, heat-inactivated

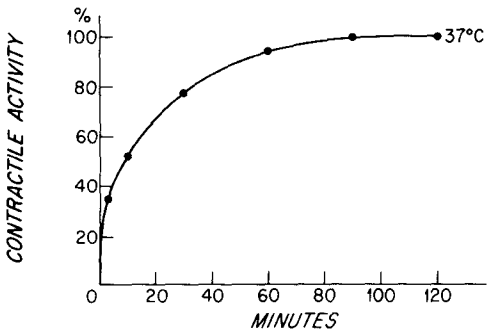

FIG. 1. Time-course of generation of contractile activity from crude neutrophil extract and heat-inactivated plasma. 1,624 ng BK Eq/ml of contractile activity were present at 120 min and represent 100% of available activity.

plasma and crude neutrophil extract was 7.8–8.0, representing the upper range of a broad pH optimum of pH 5.0–8.2.

Neutral Peptide.—

Physical characteristics: In order to achieve partial purification and to determine the approximate size of the contractile principle, 10,000 ng BK Eq were generated from 4.5 ml of heat-inactivated plasma by incubation with 500 μl of neutrophil extract from 5×10^7 neutrophils for 60 min at 37°C. The activity recovered from the supernatant after 80% ethanol precipitation was flask evaporated, resuspended in 1.0 ml of 0.01 M acetic acid and filtered through a previously calibrated Sephadex G-15 column equilibrated in 0.01 M acetic acid. Individual fractions were bioassayed, and a single peak of contractile activity containing 90% of that applied was found (Fig. 2) corresponding to the bradykinin marker at 65% bed vol and consistent with a mol wt of approximately 1,000.

In order to define the charge of the contractile principle, 1,000 ng BK Eq of Sephadex G-15 purified material were subjected to isoelectric focusing in 4% acrylamide gels using pH 3.0–10.0 and subsequently pH 6.0–8.0 ampholytes (9). The gels were sliced into 5-mm long cross-sections, washed, eluted in 500 μl distilled water for 12 h at 4°C, and bioassayed after determining the pH. Activity was found only in fractions which corresponded to pH 7.0–8.0 with pH 3.0–10.0 ampholytes and to pH 7.3–7.5 with pH 6.0–8.0 ampholytes; recovery was 35–40% of the activity applied.

The neutral isoelectric point of the contractile activity clearly distinguished it from the cationic peptide, bradykinin. In order to explore further the functional homogeneity of the Sephadex G-15 purified principle, 100 μl of 0.01 M acetic acid containing 1,000 ng BK Eq of activity were subjected to descending paper chromatography in butanol:acetic acid:water (4:1:5) for 12 h at room temperature with DNP-lysine as a reference marker (10). The paper strip was cut transversely into 2-cm pieces and each was eluted with 3.0 ml 0.01 M

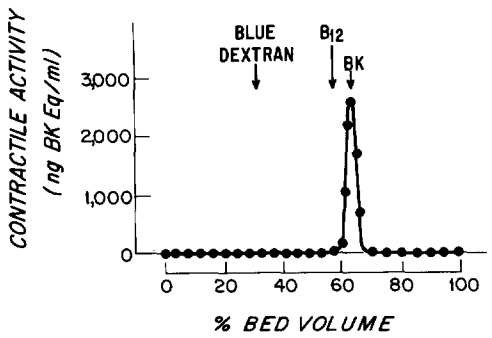

FIG. 2. Sephadex G-15 chromatography of contractile activity.

acetic acid. Eluate fractions were lyophilized, resuspended in 1 ml Tyrode's solution, and bioassayed. Activity was found in a single peak, representing 10% of the starting activity, which migrated 4–8 cm from the origin, and had an Rf of 0.25 with respect to DNP-lysine.

Susceptibility to inactivation by chymotrypsin and trypsin: In order to determine if the active principle was a peptide, its susceptibility to chymotrypsin and trypsin inactivation was compared to that of bradykinin. 200 ng BK Eq of Sephadex G-15 purified contractile principle or 400 ng of bradykinin in 100 μl of Tris:acetate pH 7.8 buffer were incubated with 1.0 mg chymotrypsin or 1.0 mg trypsin in 900 μl of the same buffer for 30 min at 37°C. The reaction mixtures and control solutions of contractile principle and bradykinin were boiled for 15 min. Direct bioassay of the mixtures revealed that both enzymes completely inactivated the neutral contractile principle while bradykinin was susceptible only to chymotrypsin. Taken together with the physical chemical data, these studies permit designation of the active principle as neutral peptide.

Biologic activity: The contraction of the atropinized and antihistamine-treated guinea pig ileum elicited by crude or partially purified neutral peptide was slow and more reminiscent of the action of SRS-A than of histamine or bradykinin. Repeated maximal contractions were not associated with tachyphylaxis. A concentration of neutral peptide equal to 13 ng BK Eq as assayed on the guinea pig ileum recorded less than a 0.4 ng BK Eq on the estrous rat uterus. Thus, neutral peptide did not have the contractile characteristics of a kinin peptide.

Neutrophil Protease.—

Inhibition: A 100-μl portion of crude neutrophil extract from 10^7 cells was subjected to heating at 60°C for 15 min before incubation with 900 μl of heat-inactivated plasma for 5 min at 37°C. Unheated, crude neutrophil extract generated 500 ng BK Eq of neutral peptide, while the heated extract had no activity.

In order to characterize further the heat-labile neutrophil extract protease,

100-μl portions of neutrophil extract from 10^7 neutrophils were exposed to varying doses of DFP in 10 μl of 0.0035 M PO_4-buffered 0.15 M NaCl (pH 7.4) for 60 min at 37°C followed by extensive dialysis in the same buffer to remove residual DFP. 50 μl of each reaction mixture were then mixed with 450 μl of heat-inactivated plasma, incubated for 5 min at 37°C, placed on ice, and the samples directly bioassayed for neutral peptide. Complete inhibition of the neutral peptide-generating activity occurred with 5×10^{-4} M DFP consistent with a serine at the active site of the heat labile protease in crude neutrophil extract.

The DFP-sensitive neutrophil protease was further characterized by interacting 50 μl of crude extract from 5×10^6 neutrophils with 50 μl of equimolar quantities of lima bean, soybean, pancreatic, and ovomucoid trypsin inhibitors for 15 min at 37°C before addition of 450 μl of heat-inactivated plasma. 5 min later, the reaction mixtures were placed on ice and the neutral peptide generated was directly bioassayed. At a concentration of 5×10^{-7} M, inhibition achieved with lima bean trypsin inhibitor was 85%; with soybean trypsin inhibitor, 35%; with pancreatic trypsin inhibitor, 10%; and with ovomucoid trypsin inhibitor, 0% (Fig. 3).

The susceptibility of the neutrophil serine protease to inhibition by 5×10^{-7} M lima bean and soybean trypsin inhibitors prompted an analysis of the effect of the major human plasma trypsin inhibitor, α-1-antitrypsin. 50-μl portions of extracts from 5×10^6 neutrophils were preincubated for 15 min at 37°C with 1.1–110 μg of highly purified α-1-antitrypsin or 80 μg of α-2-macroglobulin in 100 μl 0.0035 M PO_4-buffered 0.15 M NaCl (pH 7.4). These mixtures were then incubated with 450 μl heat-inactivated plasma for 5 min at 37°C and placed on ice before bioassay. Fig. 4 demonstrates a linear relationship between the percent inhibition of neutral peptide generation and the log of the α-1-antitrypsin dose. The dose of α-1-antitrypsin capable of inhibiting the neutrophil protease from 5×10^6 cells by 50% was 11 μg, which is less

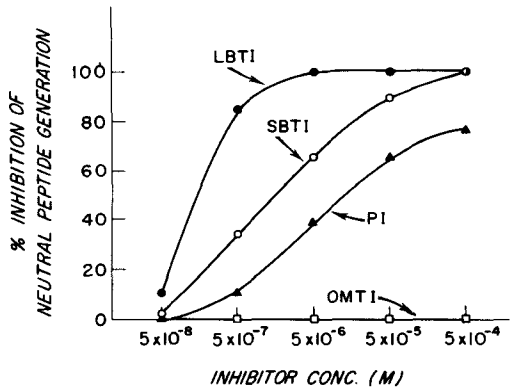

FIG. 3. LBTI, SBTI, PI, and OMTI inhibition of neutral peptide-generating activity in crude neutrophil extract. The untreated extract generated 330 ng BK Eq/ml neutral peptide.

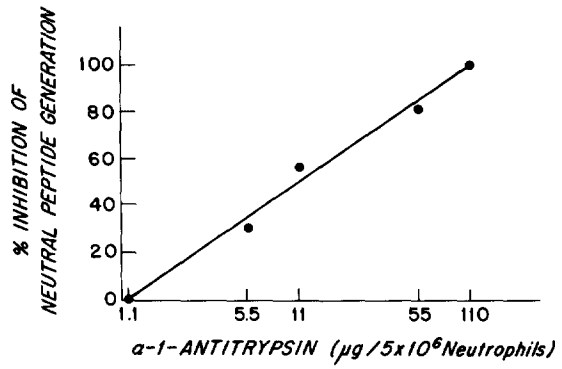

FIG. 4. α-1-antitrypsin inhibition of neutral peptide-generating activity in crude neutrophil extract. The untreated extract produced 462 ng BK Eq/ml neutral peptide.

than 1.0% of the normal plasma α-1-antitrypsin level (11). α-2-macroglobulin had no detectable effect. The functional integrity of α-2-macroglobulin was established by total inhibition of the capacity of kallikrein to generate 500 ng of bradykinin from 1.0 ml of heat-inactivated plasma (4).

The significance of plasma inhibitors other than α-1-antitrypsin was assessed by interacting crude neutrophil extract with the plasma of a patient homozygous for α-1-antitrypsin deficiency (ZZ) with 25% of the normal level (11) and with the plasma of the patient with no detectable (null) α-1-antitrypsin (6). 50 μl of neutrophil extract were incubated for 30 min at 37°C with 450 μl of whole unheated α-1-antitrypsin-deficient plasma (null), α-1-antitrypsin deficient plasma (ZZ) or whole unheated normal or heat-inactivated plasma. The reaction mixtures were placed on ice and bioassayed. 500 ng BK Eq of activity were generated from (null) α-1-antitrypsin deficient plasma or heat-inactivated normal plasma while no activity appeared in α-1-antitrypsin deficient (ZZ) or unheated normal plasma. Thus, α-1-antitrypsin appeared to be the only significant inhibitor of the neutrophil protease.

Subcellular localization: 1.6×10^8 purified neutrophils and 1.2×10^8 mononuclear leukocytes were homogenized in 20% sucrose. 25% by vol of each cell type was assayed as broken cells to determine total available subcellular enzyme markers, neutral peptide-generating activity and neutral peptide-destroying activity. 75% of each broken cell type was subjected to stepwise, differential centrifugation at 100 *g*, 400 *g*, and 17,000 *g*. Precipitates were washed, resuspended in 0.5 ml Hanks' solution, and sonicated for 3 min at 4°C. 100-μl portions of each fraction were assayed for neutral peptide-generating or destroying activity and 50-μl portions were assayed for each of the subcellular enzyme markers, lactic dehydrogenase (12), alkaline phosphatase (13), and β-glucuronidase (14). 200-μl portions of each fraction were dialyzed for 72 h at 4°C against 0.01 M Tris-buffered 0.15 M NaCl (pH 7.4) before assaying for 5′-nucleotidase (15). Neutral peptide destruction was assessed by addition of 100 ng BK Eq of Sephadex G-15 purified neutral peptide to 100 μl

of each subcellular fraction, incubation for 30 min at 37°C, and immediate bioassay of iced mixtures. 90% of the starting neutral peptide-generating activity was associated with 55% of the membrane marker 5′-nucleotidase (16) in the 100 *g* precipitate fraction while less than 5% of the activity was present in each of the 400 *g* or 17,000 *g* precipitate fractions and none in the 17,000 *g* supernate (Fig. 5). β-glucuronidase and alkaline phosphatase, which are lysosomal markers (17), were most prominent in the 17,000 *g* precipitate while lactic dehydrogenase, a cytoplasmic marker (17), was principally localized in the 17,000 *g* supernate. The 400 *g* fraction contained only isolated nuclei by phase microscopic examination. Ability to destroy neutral peptide was absent in the broken cell preparation but was associated with the 100 *g* subcellular

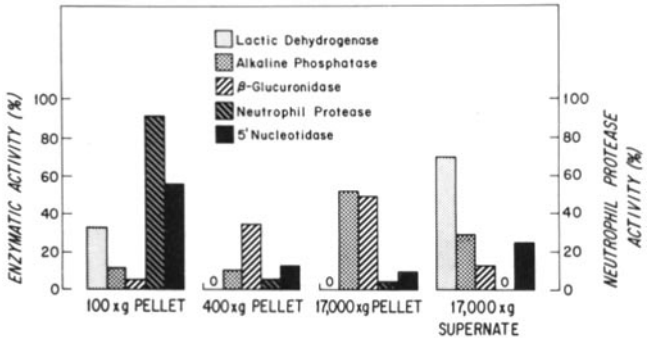

FIG. 5. Stepwise, differential centrifugation of purified, disrupted human neutrophils and assay for neutral peptide-generating activity and subcellular fraction markers. Th teotal activity was 586 mg NADH utilized/h at pH 7.5, 25°C of lactic dehydrogenase, 357 μM *p*-NO_2 phenol generation at pH 10.5, 37°C of alkaline phosphatase, 163 μg phenolphthalein generated/h at pH 6.8, 37°C of β-glucuronidase, 246 μg phosphate generated/h at pH 7.5, 37°C of 5′-nucleotidase, and 5,010 ng BK Eq neutral peptide/1.2 × 10^6 neutrophils of neutrophil protease.

fraction. Mononuclear cells manifested no neutral peptide-generating or destroying activity.

The finding that the neutral peptide-generating activity was present only with the 100 *g* fraction suggested an association with the membrane or some other rapidly sedimenting debris. Accordingly, 1.0 ml of the crude neutrophil extract routinely used as a source of neutral peptide generating activity was subjected to gel filtration through Sepharose 4B equilibrated in 0.0035 M PO_4 buffered 0.15 M NaCl (pH 7.4). 200 μl of each column fraction was incubated with 200 μl of heat-inactivated plasma for 10 min at 37°C before placing the samples on ice and direct bioassay of neutral peptide generated. The capacity to generate neutral peptide was present only in excluded fractions, in association with 64% of the 5′-nucleotidase activity, and with a recovery of 90% of starting neutral peptide-generating activity.

Since the neutral peptide-generating activity was associated with a large, rapidly sedimenting structure, an attempt was made to inhibit the activity in intact and fragmented cells with an inhibitor which might not rapidly penetrate the neutrophil. Intact neutrophils were exposed to α-1-antitrypsin, 260 μg/10^8 cells in 1.0 ml of Hanks' solution, for 60 min at 37°C before repeated washing and resuspension in 1.0 ml Hanks' solution. 50% of the α-1-antitrypsin treated and untreated control cells were then fragmented by freeze-thawing and sonication. 100 μl of either intact cells or extract were incubated with 900 μl of heat-inactivated plasma for 5 min at 37°C, before addition of 4.0 ml cold ethanol, flask evaporation of the supernate, resuspension in 1.0 ml distilled water and bioassay. The neutral peptide-generating ability of intact cells fell 54% from 125 to 58 ng BK Eq/ml and that of the subsequently fragmented cells 46%, from 838 to 450 ng BK Eq/ml after pretreatment of intact cells with α-1-antitrypsin.

Plasma Substrate for the Neutrophil Protease.—100 ml fresh plasma which had been dialyzed for 8 h at 4°C against 5 liters 0.0075 M Tris:HCl, pH 8.0, with two changes of buffer and centrifuged for 20 min at 4°C at each buffer change to remove precipitated protein, were applied to a QAE Sephadex A-25 column equilibrated with 0.0075 M Tris:HCl, pH 8.0. The column was washed overnight with starting buffer and a 0.3 M linear sodium chloride gradient applied. 500-μl portions of each fraction to be bioassayed for neutral peptide activity were heated for 2 h at 61°C before interaction with 50 μl crude neutrophil extract for 30 min at 37°C. A single peak of plasma substrate for neutrophil protease was detected late in the albumin region at 12.0 mS following the kininogen peak at 10.9 mS (Fig. 6) with a 61% recovery of starting activity. α-1-antitrypsin, as detected by Ouchterlony immunodiffusion, chromatographed in the albumin region and overlapped the neutrophil protease plasma substrate peak. Fractions 120–140, containing neutrophil protease plasma substrate,

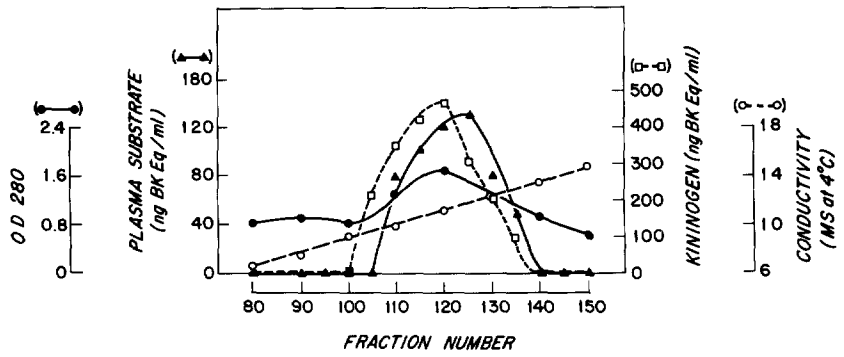

FIG. 6. Plasma substrate for neutrophil protease and kininogen in the albumin peak of plasma chromatographed on QAE Sephadex A-25. Kininogen was assayed functionally by trypsin cleavage and release of bradykinin (18).

were pooled and concentrated to 10.0 ml and a 3.0-ml portion was filtered through Sephadex G-150 equilibrated in 0.0035 M PO_4-buffered 0.15 M NaCl (pH 7.4). Heated fractions were assayed for substrate as above, and a single peak which corresponded to a mol wt of 85–95,000 was detected preceding the main protein peak. Kininogen, as assayed functionally by trypsin cleavage and release of bradykinin (18), was not recognized in the filtration step.

The isolation procedure for substrate was repeated in identical fashion using 100 ml of plasma from the (null) α-1-antitrypsin-deficient patient. QAE Sephadex A-25 chromatography demonstrated a single peak of plasma substrate for neutrophil protease which eluted after kininogen at 12.0 mS, and could be identified with or without heating. Heating, however, was associated with a twofold increase of neutral peptide generation throughout the entire substrate peak, indicating that heating either increased substrate susceptibility or destroyed an inactivator of the neutral peptide since α-1-antitrypsin was absent from the starting material. Sephadex G-150 gel filtration of the pooled, concentrated substrate-containing fractions (Fig. 7) revealed an ascending region in which neutral peptide generation was not augmented by the heat-inactivation effect and a descending region in which heating increased neutral peptide generation. The presence of a heat-labile neutral peptide inactivator in the descending region was established by incubating 100 ng BK Eq of Sephadex G-15 purified neutral peptide with 200 μl of unheated substrate-containing

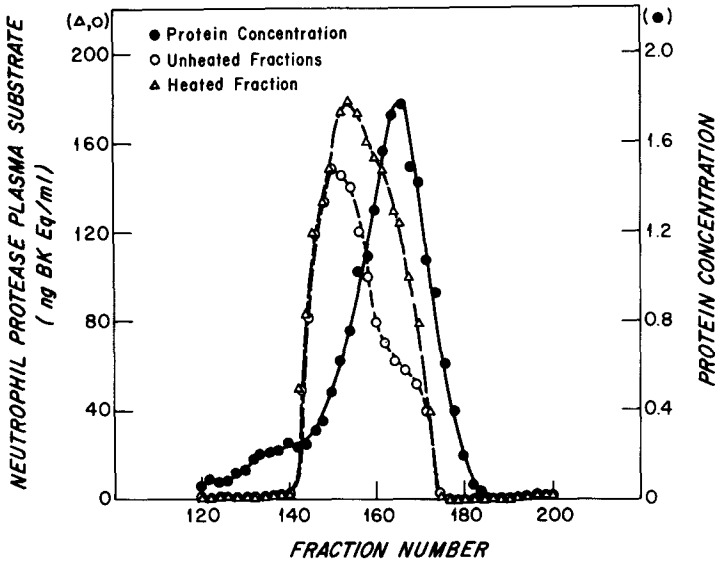

FIG. 7. Sephadex G-150 filtration of plasma substrate for neutrophil protease obtained by QAE Sephadex chromatography of plasma from the (null) α-1-antitrypsin-deficient patient. Values for protein concentration are net absorption at 600 nM. The column was previously calibrated with aldolase, human serum albumin, and chymotrypsinogen which peaked in fraction 132, 171, and 193 respectively.

fraction for 30 min at 37°C. Complete inactivation of neutral peptide occurred in those fractions of substrate whose capacity to generate neutral peptide was augmented by heating, while there was no inactivation in the substrate fractions unaffected by heating.

DISCUSSION

A biologically active, low molecular weight neutral peptide mediator cleaved from a plasma protein substrate by a serine protease associated with the neutrophil membrane has been recognized. The formation of neutral peptide is exquisitely controlled by the action of α-1-antitrypsin on the neutrophil protease, and the peptide mediator is inactivated by a plasma factor separable from the other reactants. The neutrophil protease responsible for neutral peptide generation is distinguished from α-1-antitrypsin inhibitable lysosomal esterases such as neutral leukocyte protease (19), elastase (20), and the kinin-forming enzyme (3) by its subcellular location (Fig. 5) and from plasma kallikrein by the failure of α-2-macroglobulin to inhibit its action (4). The substrate is distinguished antigenically[2] and chromatographically from kininogen (Fig. 6), and there are critical physical, chemical, and functional differences between neutral peptide and bradykinin. This does not preclude the possibility that any of the five recognized components may participate in other reaction pathways of host defense and inflammation.

The biologically active mediator generated by the interaction of the neutrophil protease and the plasma substrate was designated neutral peptide because of its isoelectric point of 7.3–7.5 and inactivation by chymotrypsin and trypsin. Only a single functional species of neutral peptide was recognized by isoelectric focusing, descending paper chromatography, and Sephadex G-15 filtration. Sephadex G-15 filtration revealed a mol wt for neutral peptide indistinguishable from the marker nonapeptide bradykinin, approximately 1,000 (Fig. 2). Neutral peptide elicits a slow contraction of the isolated guinea pig ileum pretreated with atropine and mepyramine, has little activity on the estrous rat uterus and increases vascular permeability in guinea pig skin.

The neutrophil enzyme responsible for the generation of neutral peptide (Fig. 1) was not detectable in purified mononuclear cells, was labile to heating at 61°C for 15 min, and was a tryptic serine protease based on its inactivation by DFP (21) and inhibition by lima bean and soybean trypsin inhibitors (Fig. 3). Several lines of evidence suggest that the protease is available on the membrane possibly as an ectoenzyme. The protease activity was present only in the excluded vol of gel filtration of the crude neutrophil extract through Sepharose 4B in association with the membrane marker 5′-nucleotidase, implying association with particles of a mol wt greater than 10 million. Subcellular fractionation of purified human neutrophils located the neutral peptide-generating activity in the heaviest fraction containing predominantly fragments of cells

[2] Spragg, J. S. Unpublished observation.

with cytoplasmic membrane (Fig. 5) and failed to disclose activity with the fractions containing principally nuclei, lysosomes, or cytoplasm. Finally, α-1-antitrypsin treatment of intact neutrophils with an amount known to inhibit crude neutrophil extract by about 50% (Fig. 4) inhibited both intact cells and their broken cell product by that same percent. As α-1-antitrypsin is a plasma protein, it would not be expected to enter cells rapidly by simple diffusion and it thus seems likely that its inhibition of the neutrophil serine protease of intact cells occurs at the membrane.

The use of heat inactivation at 61°C for 2 h proved critical to the formation and stability of the neutral peptide in normal plasma and hence to the isolation of substrate from plasma. The heat-labile inhibitor of neutral peptide formation in plasma proved to be α-1-antitrypsin. α-1-antitrypsin chromatographed with substrate on anion exchange chromatography and thus only substrate isolated from a (null) α-1-antitrypsin deficient patient yielded neutral peptide without a heat inactivation step before interaction with the neutrophil protease (Fig. 7). An inactivator chromatographed with the substrate on QAE Sephadex but was distinguished on Sephadex G-150 filtration by its appearance only on the descending limb of the substrate peak (Fig. 7).

The exquisite sensitivity of the neutrophil serine protease to purified α-1-antitrypsin (Fig. 4) was recognized by the capacity of 1% of the normal plasma concentration of α-1-antitrypsin to suppress the neutral peptide-generating activity derived from the neutrophils contained in the same vol of whole blood. Indeed, neutral peptide generation in plasma occurred only with plasma of a unique patient with no detectable α-1-antitrypsin. The recognition of a neutrophil protease with α-1-antitrypsin as the only readily demonstrable control protein raises the possibility that neutrophil accumulation might overwhelm the limited levels in α-1-antitrypsin-deficient persons, thereby permitting cleavage of substrate for neutral peptide as well as other undefined protease activity.

SUMMARY

A biologically active neutral peptide mediator is cleaved from a plasma protein substrate by an α-1-antitrypsin-inhibitable serine protease apparently residing on the membrane of the human neutrophil. The peptide mediator has an approximate mol wt of 1,000, and is distinguished from the kinin peptides by a neutral isoelectric point, susceptibility to inactivation by trypsin as well as chymotrypsin and activity on the isolated, atropinized, and antihistamine-treated guinea pig ileum with relatively little action on the estrous rat uterus. The neutrophil protease is fully inhibitable by DFP, trypsin inhibitors from lima or soy bean, and α-1-antitrypsin and is associated with the high mol wt fragments of the neutrophil and not the nuclear, lysosomal, or cytoplasmic subcellular fraction. The substrate has an approximate mol wt of 90,000 and is chromatographically separable from kininogen. The exquisite sensitivity

of the neutrophil protease to α-1-antitrypsin was established both by inhibition with highly purified α-1-antitrypsin and by the inability of the protease to generate detectable neutral peptide in a homozygous (ZZ) α-1-antitrypsin-deficient patient without heat inactivation of the residual inhibitor. On the other hand, plasma from a (null) α-1-antitrypsin-deficient patient supported neutral peptide generation and revealed an additional factor which inactivated neutral peptide.

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