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FORMATION OF POLYMORPHONUCLEAR LEUKOCYTE ELASTASE:α_1 PROTEINASE INHIBITOR COMPLEX AND Aα(1–21) FIBRINOPEPTIDE IN HUMAN BLOOD STIMULATED WITH THE CALCIUM IONOPHORE A23187

A MODEL TO CHARACTERIZE INHIBITORS OF POLYMORPHONUCLEAR LEUKOCYTE ELASTASE

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Abstract—Incubation of human blood with the secretagogue A23187 resulted in the formation of increased plasma concentrations of polymorphonuclear leukocyte (PMN) elastase:α_1 proteinase inhibitor (PMNE:α_1PI) complex as well as Aα(1–21) fibrinopeptide [Aα(1–21)]. The formation of these species was both time and A23187 concentration dependent. Using a sandwich ELISA and a radioimmunoassay, we determined the comparative potencies of several compounds to inhibit the formation of PMNE:α_1PI complexes and Aα(1–21), respectively. L-658,758, a substituted cephalosporin, essentially irreversible elastase inhibitor, inhibited the formation of PMNE:α_1PI and Aα(1–21) with IC_{50} values of 38 and 15 μM, respectively. L-683,845, a monocyclic β-lactam, was much more potent against isolated PMNE than L-658,758. However in this system it was approximately equivalent to L-658,758 with an IC_{50} of 15 μM against both species. ICI-200,880, a competitive slow-binding elastase inhibitor, was significantly less potent to inhibit Aα(1–21), having an IC_{50} of 75 μM, while Declaben, a reversible noncompetitive inhibitor, was inactive at concentrations as great as 200 μM. We propose that evaluating inhibitors in the complex milieu of blood will provide a useful method to predict their therapeutic potential *in vivo*.

Key words: elastase; elastase inhibitors; antitrypsin; antiproteinase

PMNE† (EC 3.4.21.37) is a serine proteinase stored at high concentrations in the azurophilic granules of PMN [1]. While PMNE may contribute to the intracellular degradation of phagocytized materials, its release from activated PMN into extracellular spaces has been implicated in the pathogenesis of various diseases and is thought to be responsible for tissue damage [2]. The accumulation of PMN at sites of inflammation is a prominent feature of a number of inflammatory diseases. PMNE degrades a large array of connective tissue and soluble proteins including elastin, several types of collagen, fibrinogen, proteoglycans, histones, hemoglobin and various blood clotting factors. When present extracellularly, the activity of this proteinase is controlled by natural inhibitors including α_1PI, α_2M and SLPI. The concept that tissue damage is the result of an imbalance between free elastase and natural inhibitors has been referred to as the proteinase–antiproteinase hypothesis [3]. Such an imbalance may favor proteinase attack on various connective tissue and matrix proteins and is thought to contribute to diseases such as pulmonary emphysema, chronic bronchitis, cystic fibrosis, adult respiratory distress syndrome (ARDS) and consumption coagulopathies associated with Gram-negative sepsis or leukemias [4, 5].

In plasma, approximately 90% of PMNE is bound in an inactive complex with α_1PI, and the remainder is complexed to α_2M [6]. Elevated concentrations of PMNE:α_1PI complex have been detected in plasma from patients with a variety of inflammatory diseases including rheumatoid arthritis, pancreatitis, ulcerative colitis, cystic fibrosis, ARDS, and both bacteremic and nonbacteremic septicemia [7, 8]. Elevated plasma concentrations of PMNE:α_1PI complex have been reported from patients undergoing renal hemodialysis [9–11], and increased amounts of this complex have been found in the sera from clotting blood [12].

A number of investigators have demonstrated that fibrinogen is a substrate for PMNE [13–17]. A primary PMNE cleavage site on fibrinogen is at the Aα(Val^{21}-Glu^{22}) position on the Aα chain releasing a 21-residue peptide referred to as Aα(1–21) [15–17].

Elastase released from PMN may cause substrate

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† Abbreviations: PMN, polymorphonuclear leukocyte; PMNE, PMN elastase; α_1PI, α_1 proteinase inhibitor; Aα(1–21), Aα(1–21) fibrinopeptide; α_2M, α_2-macroglobulin; SLPI, secretory leukoproteinase inhibitor; RIA, radioimmunoassay; and LDH, lactate dehydrogenase.

degradation in the presence of naturally occurring inhibitors. Elastase-mediated fibrinogenolysis by activated PMN occurs in the presence of physiological concentrations of antiproteinases [18]. Weiss and Regiani [19] similarly showed that PMN degraded subendothelial matrices in the presence of α_1PI. However, SLPI, a smaller serine proteinase inhibitor found in mucous secretions, effectively inhibited the breakdown of radiolabeled fibronectin and elastin mediated by stimulated PMN adhered to these protein matrices [20].

We have shown that PMN adhered to a matrix of fibrinogen can be stimulated to secrete PMNE, resulting in the degradation of the protein with the formation of the specific fibrinogen cleavage product Aα(1–21) [21]. Under these *in vitro* conditions, α_1PI (mol. wt = approx. 54 kDa) was not as effective in inhibiting Aα(1–21) production (IC_{50} = 220 nM) as compared with SLPI (mol. wt = approx. 12 kDa) (IC_{50} = 85 nM) [22]. These observations suggest that in certain environments small molecular weight natural inhibitors may be more effective than the larger natural inhibitor α_1PI in inactivating PMNE. Therefore, it is plausible that low molecular weight synthetic PMN elastase inhibitors may be more effective in inhibiting proteolysis caused by activated PMN than are the natural proteinase inhibitors. Thus, low molecular weight selective inhibitors of PMNE may be efficacious therapeutic agents if they can be delivered to inflammatory sites containing degranulating PMN.

In these studies we describe an *in vitro* system to evaluate the potency of PMNE inhibitors in the milieu of human blood containing physiological concentrations of natural inhibitors α_1PI and α_2M, as well as plasma protein substrates of PMNE and plasma proteins that may bind or inactivate the putative inhibitor. Incubation of blood with the calcium ionophore A23187 stimulates the secretion of PMNE from PMN. Although human blood contains a large molar excess, approximately 1000-fold of natural inhibitors over that of PMNE, the enzyme is not inhibited completely at the time of its release from degranulating PMN. This activity can be monitored by the cleavage of Aα(1–21) from the N-terminal of the Aα chain of fibrinogen. Subsequent to hydrolyzing fibrinogen in the plasma, the PMNE is inactivated by α_1PI forming PMNE:α_1PI complex.

Using this system, we have carried out a comparative evaluation of representatives of different classes of synthetic inhibitors. These include a cephalosporin PMN elastase inhibitor L-658,758 [23–26], a time-dependent inhibitor with K_{obs}/K_i of 4200 $M^{-1}sec^{-1}$; a monocyclic-β-lactam inhibitor L-683,845 [27, 28], also a time-dependent inhibitor with K_{obs}/K_i of 848,000 $M^{-1}sec^{-1}$; ICI-200,880 [29], a competitive slow-binding inhibitor, and Declaben [30], a reversible non-competitive inhibitor.

L-658,758, L-683,845 and ICI-200-880 inhibited fibrinopeptide Aα(1–21) and PMNE:α_1PI complex formation with different levels of potency. Declaben was inactive.

MATERIALS AND METHODS

Materials. Human PMNE was obtained from Elastin Products, Pacific, MO. Human α_1PI was from Athens Research and Technology, Athens, GA.

L-658,758 (2,3-dihydro-6-[3-(2-hydroxymethyl)-phenyl-2-propenyl]-5-benzofuranol), L-683,845 (4-((1-(((1-(*R*)-(5-benzofuranyl)butyl)amino)carbonyl)-3,3-diethyl-4-oxo-2-(*S*)azetidinyl)oxy)-benzene acetic acid, ICI-200,880 [4-(4-chlorophenyl sulfonylcarbamoyl)benzoyl-L-valyl-L-prolyl-1-(*RS*)-(1-trifluoroacetyl-2-methylprolyl)amide] and Searle's Declaben (SC-39026), 2-chloro-4-(1-hydroxy-octadecyl)benzoic acid, were prepared by Dr. C. Dorn of the Medicinal Chemical Research Department of our laboratories. The structures of these compounds are shown in Fig. 1.

Incubation of human blood with A23187. Blood was obtained by antecubital venipuncture from informed adult volunteers and collected into heparin, 50 U/mL. Aliquots (1 mL) were preincubated for 10 min at 37° with PMNE inhibitors prior to the addition of A23187, usually 100 μM. After 30 min the incubations were terminated by centrifugation at 12,000 *g* for 5 min. The plasma fraction was removed and analyzed for PMNE:α_1PI complex formation by a sandwich ELISA. The ELISA was modified from the methodology described by Neumann *et al.* [31]. The amounts of lactic dehydrogenase in the plasma were determined by the method of Morgenstern *et al.* [32], adapted to automated colorimetry in 96-well plates. Plasma PMNE concentrations were determined by a competitive RIA using a polyclonal rabbit antiserum directed against isoform 4 of human leukocyte elastase [24].

Determination of Aα(1–21). Aliquots (200 μL) of plasma were treated with 300 μL of cold acetone. After removing the denatured protein by centrifugation at 12,000 *g* for 5 min, the amounts of Aα(1–21) in the aqueous-acetone supernatant fluids were determined by competitive RIA.

Briefly, a rabbit polyclonal antiserum, designated R20, was developed using H_2N-Cys-Nle-Aα (Gly^{17}-Pro^{18}-Arg^{19}-Val^{20}-Val^{21}-OH) coupled to BSA as the immunogen. The assay was found to have a sensitivity limit of 0.04 pmol/tube in a competitive RIA format using ^{125}I-Tyr-Aα(1–21) as the radioactive probe. The assay has been described in detail by Mumford *et al.* [17, 33].

The recovery in the plasma of exogenously added Aα(1–21) to aliquots of human blood was approximately 50%. The intra-assay variation was 11% and the inter-assay variation was 18% (N = 12).

Determinations of PMNE. Aliquots of plasma (10–50 μL) were analyzed for PMNE by a competitive RIA. The assay system detects PMNE both as the free enzyme and as PMNE complexed to α_1PI or to L-658,758 and L-683,845 with a limit of sensitivity of approximately 0.1 pmol/assay tube.

A polyclonal rabbit antiserum was prepared by immunization of rabbits with isozyme 4 of PMNE, prepared by FPLC of PMNE isolated from cystic fibrotic sputum (Elastin Products). PMNE was radioiodinated by the chloramine T procedure with slight modifications of the method described by Hunter and Greenwood [34]. The ^{125}I-PMNE was separated from unreacted $Na^{125}I$ by size exclusion

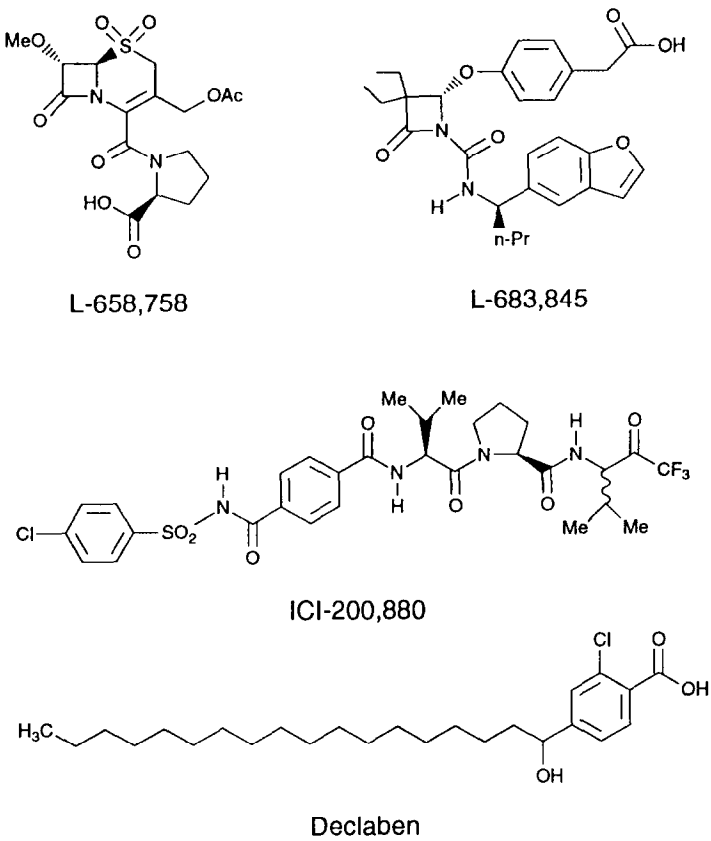

Fig. 1. Structures of PMNE inhibitors discussed in this manuscript.

chromatography on Sephadex G-50 eluted with the RIA buffer consisting of 10 mM $NaHPO_4$, pH 7.4, 0.6 M NaCl, 2 mM EDTA, 0.01% thimerisol, 2% heat-inactivated horse serum and containing 130 mg sodium heparin and 20 U aprotinin/mL. The RIA incubations contained 50 μL of the antiserum diluted 1:8000 in the RIA buffer, 50 μL of the ^{125}I-PMNE probe (30,000 cpm/50 μL of RIA buffer), and 100 μL of samples or 100 μL of PMNE standard (0.027 to 7.0 pmol/100 μL RIA buffer). The samples were incubated at 4° for more than 6 hr. The unbound ^{125}I-probe was separated from antibody-bound radioactivity by incubation at room temperature with 0.5 mL of a suspension of donkey anti-rabbit IgG magnetic beads (Amersham). After 30 min the samples were placed onto a magnetic rack. After approximately 5 min the supernatant fluid was decanted, and the radioactivity associated with the beads was determined.

Determination of PMNE:α_1PI complex. The plasma concentrations of PMNE:α_1PI complex were measured by an ELISA. Immunolon 2, 96-well microtiter plates were coated with 200 μL of sheep anti-human PMN elastase (ICN) diluted 1:1000 in Voeller's buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 3 mM NaN_3 in H_2O, pH 9.6). The plates were incubated overnight at 4° in a humid box. Prior to use, the plates were washed three times with wash buffer (0.075% Tween 20 in a phosphate-buffered saline solution containing 137 mM NaCl, 1.5 mM KH_2PO_4, 10.8 mM $Na_2HPO_4 \cdot 7H_2O$, and 2.7 mM KCl, pH 7.4).

Human PMNE:α_1PI complexes were prepared by incubating 8 nmol PMNE with 17 nmol α_1PI in 1.2 mL wash buffer at room temperature for 30 min. Standard PMN elastase:α_1PI complex or samples were diluted in wash buffer containing 0.1% BSA and added to the plates. After 1 hr at room temperature, the plates were washed again and 100 μL rabbit anti-human α_1PI (Behring Diagnostics, La Jolla, CA) diluted 1:500 in wash buffer was added. After another hour at room temperature the plates were washed, and 100 μL of goat anti-rabbit IgG conjugated to horseradish peroxidase (Calbiochem, La Jolla, CA) diluted 1:1000 in wash buffer was added. After 60 min, the plates were washed again, and a 100-μL substrate/chromophore solution [10 mg *o*-phenylenediamine-dihydrochloride (Sigma) dissolved in 1 mL methanol and added to 100 mL of water containing 10 μL of 30% H_2O_2 (Fisher)] was added. Color development was allowed to proceed for 15 min. The reaction was terminated by the addition of 50 μL of 2 N H_2SO_4, and the absorbance at 490 nm was determined. This ELISA has been automated on a Beckman Instruments Biomek 1000 workstation. The amounts

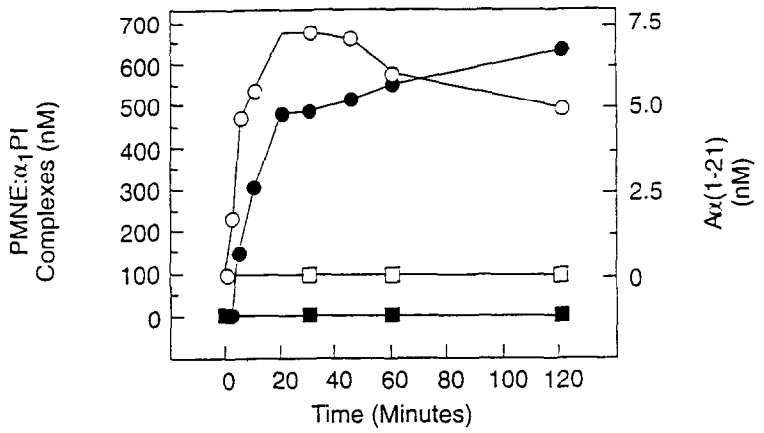

Fig. 2. Time-dependent formation of PMNE:α_1PI complexes and Aα(1–21) in human blood stimulated with A23187. Aliquots of heparinized human blood were incubated at 37°, in the presence of 100 μM A23187 or DMSO for the times indicated. The incubations were terminated by centrifugation. Plasma complex levels and Aα(1–21) concentrations were determined. The data are the means of 4 determinations. The standard deviations were less than 15%. Key: (○) Aα(1–21) and (●) PMNE:α_1PI complexes stimulated with A23187; (□) Aα(1–21) and (■) PMNE:α_1PI complexes nonstimulated.

of complex present in samples were interpolated from a standard curve of PMNE:α_1PI complex using the Beckman Immunofit program.

The recovery in the plasma of exogenously added PMNE:α_1PI complex to aliquots of human blood was 83%. The intra-assay variation was 9% and the inter-assay variation was 15%.

Polyacrylamide gel electrophoresis. Polyacrylamide precast 12% Mini-Protean II Ready Gels were purchased from BioRad (Richmond, CA). The samples were prepared under reducing conditions by the method of Laemmli [35]. Electrophoresis was performed as described for the Mini-Protean II system purchased from Bio-Rad.

PMNE (5 nmol) was preincubated in the presence or absence of 50 nmol L-658,758 in 50 μL wash buffer at room temperature prior to the addition of 6 nmol α_1PI to a final volume of 150 μL. Aliquots were mixed with an equal volume of mercaptoethanol reducing sample buffer and incubated at 100° for 2 min prior to applying 5 μL onto the gel.

RESULTS

The calcium ionophore A23187 (100 μM) stimulated the formation of PMNE:α_1PI complex and Aα(1–21) in human blood in a time-dependent manner. Maximal levels of both Aα(1–21) (7.5 nM)

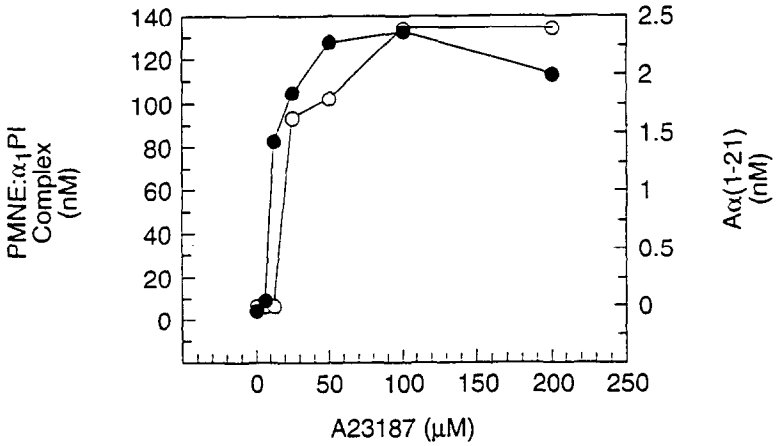

Fig. 3. Concentration-dependent A23187-stimulated PMNE:α_1PI complex and Aα(1–21) formation in human blood. Aliquots of heparinized human blood were incubated for 30 min at 37° in the presence of various concentrations of A23187. Plasma complex levels and Aα(1–21) formation were determined after centrifugation. The data are the means of 6 determinations. The standard deviations were less than 15%. Key: (○) Aα(1–21) and (●) PMNE:α_1PI complexes.

Table 1. PMNE release upon A23187 stimulation of blood without concomitant increase in plasma LDH levels

Treatment	LDH ($O.D._{450}$/mL plasma)	PMNE:α_1PI complex (nM)
DMSO	50.4 ± 3.7	4.9 ± 0.9
A23187	45.6 ± 5.8	214.9 ± 32.7
Sonication	2460 ± 72	262.0 ± 14.9

Aliquots of human blood (1 mL) were incubated in the presence or absence of 100 μM A23187 for 30 min at 37°. Plasma was prepared and LDH levels were determined and compared with plasma from sonicated controls. Data are means ± SD, N = 3.

and PMNE:α_1PI complex (> 650 mM) were observed at about 30 min after addition of A23187 (Fig. 2). No endogenous Aα(1–21) peptide was detected in the plasma from blood incubated with DMSO, the solvent for A23187, incubated at 37° over the time period of this experiment.

Although not apparent from Fig. 2, there was a small but significant increase in plasma PMNE:α_1PI complex levels (2.2 nM at 0 min increased to 6.8 nM after 120 min) in the absence of A23187, presumably due to spontaneous activation of PMN with the subsequent release of PMNE. Concentrations of 1–3 nM PMNE:α_1PI complex are routinely detected in plasma from blood collected in heparin but not incubated.

PMNE:α_1PI complex and Aα(1–21) formation were also dependent on the A23187 concentration (Fig. 3). Maximal plasma concentrations of approximately 2.5 nM Aα(1–21) and 130 nM PMNE:α_1PI were formed when blood was incubated with 100 μM A23187 for 30 min (Fig. 3).

Little or no increase in LDH levels was noted due to the treatment with A23187, suggesting that the formation of PMNE:α_1PI complex and Aα(1–21) was due to A23187-stimulated secretion of PMNE from the PMN azurophilic granule and not to cell lysis (Table 1).

L-658,758 caused a concentration-dependent inhibition of A23187-stimulated complex formation with an IC_{50} of 38.3 ± 4.4 μM (mean ± SEM, N = 3). L-658,758 similarly inhibited the formation of Aα(1–21) with an IC_{50} of 14.5 ± 3.5 μM (mean ± SEM, N = 3) (Fig. 4). L-658,758 did not affect significantly the amount of PMNE released into the plasma, as evaluated by an RIA that detects both L-658,758-bound PMNE and free-PMNE, suggesting that the compound reacted with PMNE itself rather than interfering with release of the enzyme from the azurophilic granules (Fig. 4). The maximal inhibition of complex formation by L-658,758 did not exceed 70%.

L-683,845, a monocyclic β-lactam, is much more potent than L-658,758 against PMNE. However, this compound inhibited both A23187-induced Aα(1–21) and PMNE:α_1PI complex formation in a similar manner with an IC_{50} of 15 μM (Fig. 5). L-680,833, an analogue of L-683,845, was shown previously to inhibit the formation of both Aα(1–21) and PMNE:α_1PI with an IC_{50} of 9 μM [36].

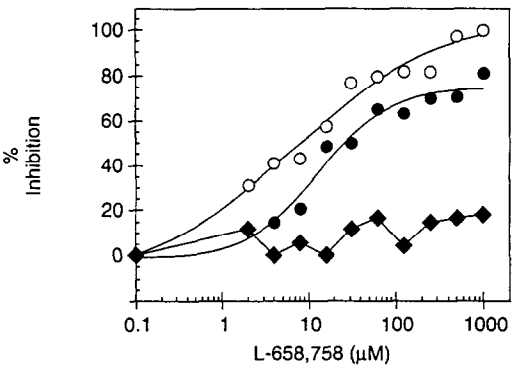

Fig. 4. L-658,758 inhibition of A23187-stimulated PMNE:α_1PI complex and Aα(1–21) peptide formation without effects on azurophilic granule release. Various concentrations of L-658,758 were preincubated with heparinized human blood for 10 min prior to the addition of 100 μM A23187. After 30 min at 37°, plasma was prepared. PMNE:α_1PI complexes, Aα(1–21), and PMNE plasma concentrations were determined. The data are the means of 3 determinations. The standard deviations were less than 20%. Key: (○) Aα(1–21), (●) PMNE:α_1PI complexes, and (◆) PMNE.

ICI-200,880 and Declaben were also evaluated and compared with L-658,758 in this system (Fig. 6). ICI-200,880, a competitive peptide trifluoromethylketone inhibitor of PMNE, inhibited A23187-induced formation of Aα(1–21) in a concentration-dependent manner with an IC_{50} of approximately 75 μM. In this experiment, L-658,758 was significantly more potent, exhibiting an IC_{50} of 12 μM. Declaben, a non-competitive and reversible PMNE inhibitor, was inactive at all concentrations evaluated.

Polyacrylamide gel electrophoresis of elastase preincubated with L-658,758 followed by addition of α_1PI also demonstrated that the inhibitor prevented the formation of PMNE:α_1PI complex (Fig. 7). Under these conditions, PMNE (5 nmol), which had been inactivated previously with L-658,758, migrated as several molecular weight species of approximately 28,000 presumably due to several isoforms of the enzyme (lane E). In contrast, native PMNE migrated at approximately 14,000, suggesting that the enzyme had undergone autolysis under the boiling/reducing conditions (lane B). When 6 nmol α_1PI (lane A) was preincubated with PMNE, several species of PMNE:α_1PI complexes were observed at 70,000–80,000 (lane C). The formation of these species was inhibited markedly by preincubation with 50 nmol of L-658,758 (lane D). The multiple high molecular weight complexes are presumably due to complexes of α_1PI with the different isoforms of PMNE.

DISCUSSION

PMNE has been proposed to participate in a variety of human diseases [4]. The mechanism of PMNE-induced tissue damage has centered on an

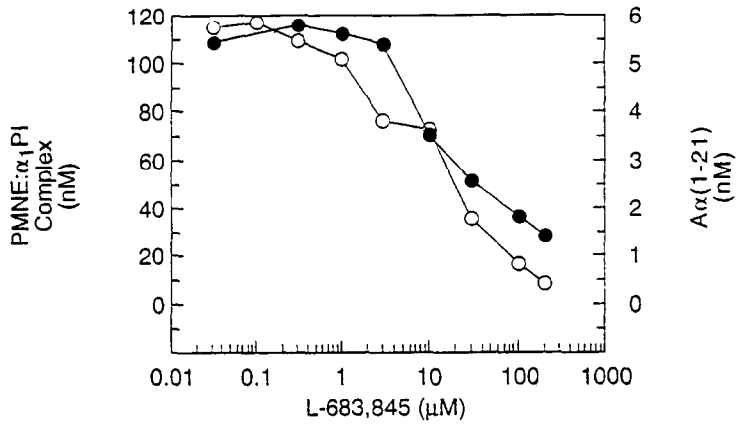

Fig. 5. L-683,845 inhibition of A23187-stimulated PMNE:α_1PI complex and Aα(1–21) peptide formation. Various concentrations of L-683,845 were preincubated with heparinized human blood for 10 min prior to the addition of 100 μM A23187. After 30 min at 37°, plasma was prepared. PMNE:α_1PI complexes and Aα(1–21) plasma concentrations were determined. The data are the means of 3 determinations. The standard deviations were less than 20%. Key: (○) Aα(1–21) and (●) PMN elastase:α_1PI complexes.

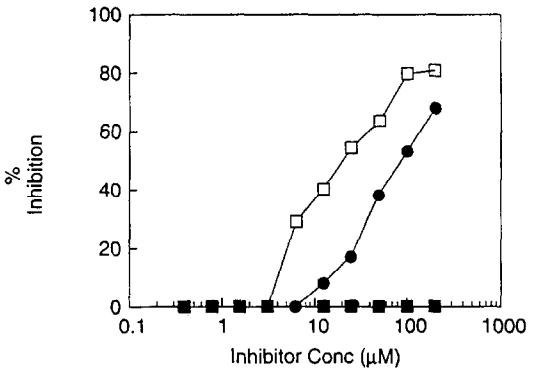

Fig. 6. Evaluation of ICI-200,880 and Declaben on A23187-stimulated Aα(1–21) formation. L-658,758, ICI-200,880 and Declaben were incubated as described in the legend to Fig. 3. The plasma concentrations of Aα(1–21) were determined by RIA as described in Materials and Methods. The data are the means of 3 determinations. Key: (□) L-658,758, (●) ICI-200,880 and (■) Declaben.

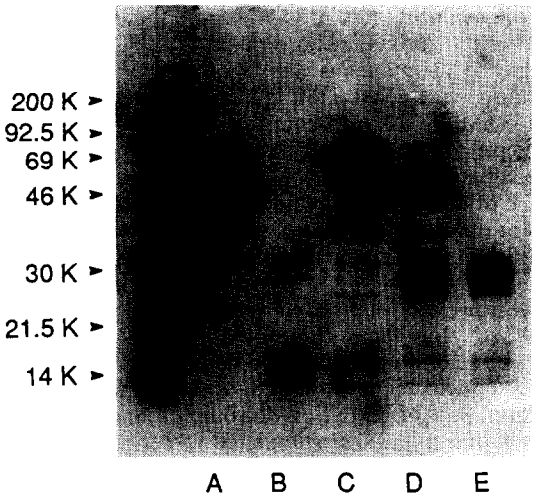

Fig. 7. Inhibition of PMNE:α_1PI complex formation by L-658,758, as determined by polyacrylamide gel electrophoresis. PMNE (5 nmol) was preincubated in the presence or absence of 50 nmol L-658,758 for 15 min at room temperature prior to the addition of 6 nmol of α_1PI. The final incubation volume was 150 μL and 5 μL was applied to the gel. Key: Lane A: α_1PI. Lane B: native PMNE. Lane C: PMNE:α_1PI. Lane D: inhibition of PMNE:α_1PI complex by L-658,758. Lane E: PMNE incubated with L-658,758.

imbalance between proteinase and antiproteinases. In 1963, Laurell and Eriksson [37] first described an inverse relationship between plasma levels of α_1PI and the severity of disease in patients with emphysema. This concept of proteinase–antiproteinase balance in extracellular environments has been a central dogma in the field of tissue damage in diseases such as emphysema.

In blood, α_1PI is the major proteinase inhibitor of PMNE. The plasma concentration of α_1PI is approximately 30–50 μM, and the deficiency of this inhibitor has been related to emphysema [37, 38]. Also, α_2M, with plasma concentrations of 2–6 μM, contributes to the proteinase–antiproteinase balance in blood [6]. However, in other environments such as the lung, it is unlikely that these plasma inhibitors play a significant antiproteinase role. In the lung, SLPI, which is secreted from serous cells of the bronchial mucous glands, Clara cells, and goblet cells, is thought to modulate and control the secreted PMNE activity [39–41].

Here we describe an *in vitro* system that we have used to evaluate the potency of PMNE inhibitors in blood. In this system we stimulated the secretion of elastase from PMN in blood by the addition of the

calcium ionophore A23187. The released PMNE caused the hydrolysis of fibrinogen prior to its inactivation by α_1PI. However, blood presents a relatively unfavorable physiological environment in which to demonstrate the biochemical efficacy of a synthetic PMNE inhibitor as it contains plasma protein, PMNE substrates such as albumin and fibrinogen, proteins from the complement cascade, as well as naturally occurring PMNE inhibitors that can compete with the compound.

L-658,758, a time-dependent cephalosporin PMNE inhibitor, with K_{obs}/K_i of 4200 $M^{-1}sec^{-1}$ to inhibit PMN elastase [24], is much slower to inhibit the enzyme than the naturally occurring inhibitor α_1PI ($k_{inact}/K_i = 6.5 \times 10^7\ M^{-1}sec^{-1}$) as reported by Beatty *et al.* [42] or ($k_{inact}/K_i = 2.2 \times 10^6\ M^{-1}sec^{-1}$) as determined by Knight *et al.* [27]. However, these studies demonstrate that in blood stimulated with A23187, L-658,758 can compete effectively with α_1PI to inhibit released PMNE. L-658,758, in a concentration-dependent manner, inhibited the formation of PMNE:α_1PI with an IC_{50} of 38 μM and also prevented A23187-induced Aα(1–21) formation. This inhibitory concentration is approximately equal to the plasma concentration of α_1PI, 30–50 μM.

The monocyclic β-lactam time-dependent PMNE inhibitor L-683,845 is an extremely fast inhibitor of PMNE, having a second order rate constant of 848,000 $M^{-1}sec^{-1}$, which approaches the rate of α_1PI. However, in the milieu of blood containing natural inhibitors of PMNE, substrates of PMNE, as well as potential inhibitor binding proteins and metabolizing enzymes, the potencies of L-658,758 and L-683,845 are similar. These observations demonstrate that rates of association with the enzyme PMNE do not necessarily predict potency in complex biological fluids such as blood.

ICI 200,880 is a potent, competitive, slow-binding peptide, trifluoromethyl ketone inhibitor of PMNE with a K_i of 0.5 nM [29]. Although demonstrating a concentration-dependent inhibition in this system, ICI 200,880 was somewhat less active (IC_{50} = 75 μM) than L-658,758 (IC_{50} = 12 μM) or L-683,845 (IC_{50} = 15 μM) to inhibit the A23187-stimulated formation of Aα(1–21) fibrinopeptide. In contrast, Declaben, a reversible non-competitive inhibitor and a chlorinated benzoic acid derivative containing a saturated hydrocarbon chain, was inactive.

The increased potency of L-658,758 and L-683,845 in relation to ICI-200,880 and Declaben may relate to any number of factors inherent to whole blood, including serum protein binding and metabolism or to the biochemical mechanism of action of the inhibitors.

We and others have demonstrated *in vitro* that PMNE can degrade various proteins in the presence of naturally occurring inhibitors [17, 18, 20]. Thus, low molecular weight, metabolically stable, selective PMNE inhibitors that could be delivered to sites of unregulated PMNE activity may be efficacious therapeutic agents. We speculate that synthetic PMN elastase inhibitors such as L-658,758 and L-683,845 may provide new and useful therapeutic modalities for the treatment of diseases characterized by PMN and PMNE involvement.

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