Neutrophils activated with serum opsonized zymosan, soluble heat-aggregated IgG, and ionophore A23187 in the presence of calcium release a material capable of initially activating factor V. Subsequent inactivation of factor V was only observed with neutrophil releasate derived from IgG and ionophore. In this study we examine the nature of this neutrophil activity and investigate its role in the regulation of factor V/Va. From early in the fractionation it was apparent that the cells contained different enzymes capable of cleaving factor V. The most active of these was isolated and found to be an isomer of human neutrophil elastase. The purified protease caused a dose-dependent activation of isolated factor V to a maximum of threefold. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, single-chain factor V was cleaved to form intermediates of 100 and 91 kilodaltons (kD). Coagulant activity correlated with the formation of a 97-kD heavy and 77-kD light chain. On prolonged incubation the formed factor Va(agon) was inactivated in association with proteolysis of the 97-kD band to smaller peptides and cleavage of the 77-kD light chain to a molecular weight of 75 kD, which is similar to thrombin-activated factor Va light chain. Neutrophil elastase also caused rapid inactivation of thrombin-activated factor V, factor Va(t). These observations suggest that elastase cleaves factor V at sites distinct from that by thrombin and therefore represents a novel factor V activation pattern. It is proposed that upon neutrophil activation elastase is secreted into the plasma milieu to initiate factor V activation. This serves to generate small amounts of thrombin that, in turn, by positive feedback fully activates factor V and thus amplifies the coagulation reaction.

In this paper we examine the nature of this neutrophil activity and its possible physiological relevance.

**MATERIALS AND METHODS**

All materials were purchased from Sigma Chemical Co (St Louis) except for platelet (General Diagnostic—Warner Lambert Co, Morris Plains, NJ); chromogenic substrates S2484, S2238, and S2222 (KabiVitrum, Stockholm); heparin (Weddel Pharmaceuticals, Sydney, Australia); 125I-Na (Amersham Corp, Sydney, Australia); iodogen and sodium dodecyl sulphate (SDS) (Pierce Chemical Co, Rockford, IL); NaCl and EDTA (Ajax Chemical Co, Sydney Australia); Ultrogel AcA 34 (LKB, Bromma, Sweden); and Sephadex C50 and Sepharose 6B CL (Pharmacia Fine Chemicals, Uppsala, Sweden).

Coagulant factors were all of human origin. Factor V, factor X and prothrombin, thrombin, and fibrinogen were purified and activated as indicated. A sheep antibody directed against human neutrophil elastase was purchased from ICN Immunobiologicals, Lisle, IL.

SDS–polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli.12 Protein assays were performed by the Bradford method13 using the Bio-Rad assay kit (Bio-rad Laboratories, Richmond, CA). Factor V coagulant activity was measured in a one-stage assay using a purified component system.14 Factor V was radiolabeled with 125I-Na by using the iodogen method (Pierce). The specific activity of the labeled material ranged from 1,000 to 1,500 cpm/ng.

Neutrophils were isolated by Ficoll-Hypaque sedimentation14 and were shown to consist of 97% neutrophils, 2% monocytes, and 1% lymphocytes as assessed by differential staining. Activation of neutrophils was performed by incubating 4 × 10⁷ neutrophils with 5 µg/mL soluble heat-aggregated IgG, 0.5 µg/mL serum opsonized zymosan, or ionophore A23187 (2.5 µmol/L) in the presence of 1.25 mmol/L CaCl₂ in a total volume of 200 mL at 37°C for 20 minutes in a Patons Aggregometer (Scarborough, Canada) with stirring. The cells were then removed by centrifugation at 10,000 g for 30 seconds and the releasate tested for its effects on factor V clotting activity. Hirudin (1 U/mL) was included to prevent any thrombin-induced activation of factor V.

Soluble heat-aggregated IgG was prepared from a 2% solution of Cohn fraction II (Sigma) in 0.15 mol/L NaCl. Two hundred...
milligrams of IgG was heated for ten minutes at 63°C followed by rapid cooling to 0°C. Ammonium sulphate was added to a final concentration of 0.62 mol/L at 4°C for 60 minutes followed by centrifugation at 2,000 g for ten minutes at 4°C. The precipitate was then dissolved in distilled water and dialyzed against 0.15 mol/L NaCl overnight at 4°C.

Serum opsonized zymosan was prepared from a modification of the method by Brentwood and Henson. Zymosan (10 mg) suspended in 0.15 mol/L NaCl was boiled for 30 minutes in a glass tube. The mixture was allowed to cool and incubated with an equal volume of normal human serum for 30 minutes at 37°C to fix complement. The serum opsonized zymosan was then washed extensively with 0.15 mol/L NaCl to remove any unbound serum proteins.

For purification of the major factor V activator/inactivator from neutrophils, all purification steps were performed at 4°C and the buffers adjusted to contain 10 mmol/L benzamidine and 2 mmol/L EDTA unless otherwise stated.

The enzyme of interest was followed throughout the purification by examining the ability of test fractions to cleave 125I–factor V as assessed on reduced SDS-PAGE with 4% stacking and 7.5% running gels. In this assay 1 U/mL 125I–factor V was incubated with 10 μL of test fraction in a total volume of 100 μL for 20 minutes at 37°C. The buffer used was 20 mmol/L Tris, pH 7.4, containing 150 mmol/L NaCl, 5 mmol/L CaCl₂, and 5 mg/mL bovine serum albumin. We concentrated our efforts on the most potent enzyme that at low concentrations cleaved factor V to form peptides similar in molecular weight to those seen in Fig 4, whereas at higher concentrations of the enzyme only peptides migrating with dye front were observed. Only fractions demonstrating complete cleavage of single-chain factor V were pooled as indicated in Figs 2A to C.

Neutrophils were collected from a patient with chronic myeloid leukemia undergoing therapeutic leukapheresis and were shown to be 98% pure with respect to neutrophils by a differential cell count. The cells were washed three times in phosphate-buffered saline, pH 7.4, followed by four cycles of freeze-thawing. The cells were then extracted for 60 minutes by using 1% Triton X-100 and the cellular debris removed by centrifugation at 100,000 g for 30 minutes.

SP-Sephadex chromatography. The supernatant from the previous step was adjusted to pH 7.4 by using 1 mol/L Tris, pH 8, and applied to a SP-Sephadex C50 column (5 x 20 cm) equilibrated in 20 mmol/L Tris-base, pH 7.4, and 200 mmol/L NaCl at a flow of 100 mL/h. Unbound protein was washed through with five-column volumes of starting buffer. The column was then developed by using a 2-L total volume of 200 to 600 mmol/L NaCl linear gradient under the same conditions.

Heparin agarose chromatography. Fractions containing the protease of interest were pooled and concentrated by ultrafiltration using a YM-10 membrane (Amicon Corp, Lexington, MA). The concentrated material was adjusted to 200 mmol/L NaCl by the addition of distilled H₂O and applied to a heparin-Sepharose 6B CL affinity column (2.5 x 20 cm) equilibrated in 20 mmol/L Tris buffer, pH 7.4, containing 200 mmol/L NaCl. Unbound protein was washed through with five-column volumes of the aforementioned buffer followed by a linear 200 to 700 mmol/L NaCl gradient of 500 mL total volume. The flow rate was 50 mL/h and 5-mL fractions were collected.

SP-Sephadex chromatography. Fractions demonstrating factor V cleaving activity were pooled, concentrated by ultrafiltration, and adjusted to a final NaCl content of 220 mmol/L with distilled H₂O. This material was applied to a second SP-Sephadex C50 column (1 x 15 cm) equilibrated in 20 mmol/L Tris buffer, pH 7.4, and 220 mmol/L NaCl at a flow rate of 11 mL/h. Bound material was eluted by using a 220 to 600 mmol/L NaCl gradient of 50-mL total volume. One-milliliter fractions were collected.

RESULTS

Neutrophils stimulated with serum opsonized zymosan, soluble heat-aggregated IgG, and the ionophore A23187 in the presence of calcium released a material that caused the activation of factor V (Fig 1). A rapid threefold increase in factor V clotting activity was observed after a two-minute incubation of the releasate resulting from the addition of ionophore to neutrophils. This was followed by progressive inactivation of the activated factor V. Serum opsonized zymosan caused the release of material from neutrophils that activated factor V to a similar degree as that by ionophore releasate; however, peak activity was achieved after ten minutes of incubation. Soluble heat-aggregated IgG-derived releasate caused a twofold activation of factor V within six minutes of incubation, which on further incubation resulted in the loss of procoagulant activity. The observed changes in factor V activity were associated with proteolysis of the single chain of factor V as assessed by PAGE (results not shown).

For isolation of the factor V activator/inactivator from neutrophils, the starting material consisted of neutrophils from a patient with chronic myeloid leukemia undergoing therapeutic leukapheresis. These cells cleaved factor V with a pattern identical to normal neutrophils. From the outset of the purification process it was apparent that the neutrophil contained several proteases that were capable of proteolysing factor V as seen as multiple cleavage patterns on SDS-PAGE. In this study, the most active protease was isolated by a combination of ion-exchange chromatography using SP-Sephadex and affinity chromatography on heparin agarose as detailed in Materials and Methods and Figs 2A to C.
Fig 2. Isolation of the factor V activator from neutrophils. The enzymic activity was followed by the degree of cleavage of radiolabeled factor V on SDS-PAGE. The major enzyme was isolated by chromatography on (A) SP-Sephadex C50, (B) heparin-Sepharose 6B CL, and (C) SP-Sephadex C50 as detailed in Materials and Methods. Fractions were pooled as indicated (---). The final purified product corresponds to the arrow in C and is shown in D on 10% SDS-PAGE under nonreducing conditions stained with Coomassie brilliant blue R.

The final product shown in Fig 2D migrated as a tightly spaced doublet with an approximate molecular weight (mol wt) of 27 kilodaltons (kD) on unreduced SDS-PAGE and 29 kD under reducing conditions. The ability of the purified product to cleave factor V was inhibited by diisopropyl fluorophosphate (0.2 mmol/L), thereby suggesting that it is a serine protease. The material cleaved the elastase-specific synthetic substrates N-BOC-L-ALA p-nitrophenyl ester and S2484 at rates similar to those observed by Visser and Blout and Kramps for human neutrophil elastase, respectively. This ability was completely neutralized after its incubation with an equimolar concentration of α1-proteinase inhibitor. Furthermore, a commercial sheep antihuman neutrophil elastase antibody inhibited both the amidolytic and the ability of the enzyme to activate factor V. These observations suggest that the purified neutrophil protease is an isomer of elastase.

To confirm that the band shown in Fig 2D is the enzyme responsible for factor V cleavage, an SDS-PAGE gel containing the purified product was cut into 1-mm slices from which the protein was eluted and subsequently incubated with radiolabeled factor V. The only detectable cleaving activity corresponded to the major band shown in Fig 2D (results not shown).

Figure 3 shows the effect of purified neutrophil elastase on factor V clotting activity. The enzyme caused a concentration-dependent activation of factor V with a maximal threefold increase in activity. SDS-PAGE analysis revealed that elastase initially cleaved factor V to form an intermediate consisting of a 100-kD heavy chain and a 91-kD light chain (Fig 4). On further incubation, these bands were proteolysed to form a 97-kD heavy and 77-kD light chain designated factor Va(e). In other experiments, we documented that maximal coagulant activity correlated with the formation of the 97-kD and 77 kD peptides. Inactivation of factor Va(e) was associated with proteolysis of the 97-kD band to smaller peptides and the cleavage of the 77-kD band to a mol wt (75 kD) similar to that of thrombin-activated factor V light chain.

These results contrast with factor V activation by thrombin, which results in a minimal tenfold enhancement in factor V activity. This more active form of factor V, herein referred to as factor Va(t), consists of a 112-kD heavy chain, a 75- to 71-kD light chain doublet, and a 150-kD activation peptide (Fig 5). Because elastase does not produce a major activation peptide that corresponds to that of thrombin-activated factor V, it is possible that this part of the factor V molecule is cleaved by the elastase.

This suggestion was examined by studying the effects of the addition of elastase to factor Va(t) as shown in Fig 5. Elastase caused rapid, progressive cleavage of the activation peptide into a band with a mol wt of 145 kD that on
continued incubation was cleaved to smaller mol wt peptides. The loss of coagulant activity was correlated with degradation of the heavy chain of the molecule. In contrast, the light chain undergoes minimal proteolysis, which indicates that there are no accessible elastase cleavage sites within this peptide. These observations confirm that elastase cleaves factor V at sites distinct from thrombin cleavage sites.

**DISCUSSION**

In the coagulation reaction, factor V acts as an essential cofactor to the serine protease factor Xa, which converts prothrombin to thrombin in the presence of a suitable surface. The platelet has been demonstrated to be an effective surface on which components of the prothrombinase complex can be assembled, and more recently other cells have been shown to mediate the formation of this complex, including monocytes, lymphocytes, and neutrophils.

Activation of factor V is necessary before it can participate in this reaction. Thrombin is the most efficient physiological activator; it cleaves factor V to a molecule, factor Va(t), that is at least ten times more active than factor V in a coagulation assay. Since thrombin is the end product of the prothrombinase reaction, it is possible that other cellular or
circulating proteases can activate factor V earlier in the coagulation reaction. The small amounts of thrombin formed can then by positive feedback fully activate factor V and thus amplify the coagulation reaction.

Kane et al have described a platelet protease that activates factor V with a pattern distinct from that caused by thrombin. This activated form of factor V is at least fourfold more active than native factor V in a coagulation assay. However, the platelet protease is cytosolic in location and not released during platelet activation, which suggests that it is unlikely to play a major role in the physiological regulation of factor V activity.

Bovine aortic endothelial cells have also been shown to contain an activator of factor V, but this is expressed only in the presence of homocysteine. It is believed that this observation may account for the increased incidence of thrombosis in patients with homocystinuria. Normal fasting individuals have undetectable levels of homocysteine, thereby placing in doubt the physiological importance of this mechanism of factor V activation in normal hemostasis.

In this study, we have observed that the stimulation of human neutrophils with serum opsonized zymosan, soluble heat-aggregated IgG, and the ionophore A23817 in the presence of calcium is associated with the release of a molecule(s) that causes the activation of factor V. The purification of this activity was complicated by the fact that the neutrophils contained a number of proteases that are capable of cleaving factor V. We have concentrated on the most active of these molecules, and our results suggest that it is an elastase. The protease was neutralized by a sheep antihuman neutrophil elastase antibody and α1-proteinase inhibitor and inactivated by diisopropyl fluorophosphate thereby suggesting that it is a serine enzyme. It cleaved synthetic substrates that are specific for elastase. On SDS-PAGE the protease migrated as a tightly spaced doublet, with an approximate mol wt of 29 kD on reduced gels and 27 kD when run unreduced. The purified material therefore appears to correspond to the elastase isomer E4 described by Baugh and Travis.

The ability of elastase to activate factor V was originally suggested by Schmidt et al. These workers demonstrated a rapid activation of factor V by a range of elastase isomers. Their studies were performed with a one-stage clotting assay for the measurement of factor V. The amount of elastase used (4 μg/mL) was far in excess of that released during normal whole blood clotting. Nevertheless, the initial activation was followed by a lengthening of the clotting time that corresponded to the inactivation of active factor V. Egbring et al noted a twofold activation of the factor V molecule upon incubation of plasma with a mixture of elastase isomers at levels seen in the plasma of patients with septicemia or acute leukemia, whereas at higher levels (400 μg/mL) only inactivation was observed.

We have demonstrated that the incubation of purified neutrophil elastase with factor V resulted in a concentration-dependent, two- to threefold activation followed by inactivation of the formed factor Va(e). A maximal increase in factor V activity correlated with the appearance of bands on SDS-PAGE different from those produced by thrombin, the platelet protease described by Kane et al, or the homocysteine-dependent factor V activator and therefore represents a novel activation pattern.

The inactivation of the formed factor Va(e) was associated with cleavage of the 97-kD peptide that on continued incubation was degraded to smaller mol wt peptides. The 77-kD peptide was also processed to a molecule with a mol wt of 75 kD that is similar to the light chain of thrombin-cleaved factor V. This product appears to be slightly more resistant to subsequent degradation by the elastase, but with prolonged incubation it is cleaved to smaller peptides that migrated with the dye front. This part of factor Va(e) may, therefore, correspond to the factor Va(t) light chain in view of the mol wt similarity and resistance to elastase cleavage. This raises the possibility that the factor Va(e) light chain, like the factor Va(t) light chain, may also act as a cofactor to thrombin by enhancing its ability to activate protein C and thus amplify the natural anticoagulant system.

Elastase has previously been shown to be released from the azurophilic granules of the neutrophil after incubation with serum opsonized zymosan, heat-aggregated IgG, and the calcium mobilizer A23187 in the presence of calcium. Therefore, our finding that the most potent protease contained within the neutrophil is an elastase is in keeping with the effects of neutrophil stimulation on factor V activity shown in Fig 1.

Our inability to demonstrate the presence of elastase-induced factor V cleavage products during whole blood clotting in vitro does not negate that this event could be physiologically important. Elastase may play a role in the activation of trace amounts of factor V very early in the coagulation reaction. The small amounts of thrombin generated may then by positive feedback fully activate factor V and thereby mask the cleavage pattern caused by elastase.

The potential of elastase to modulate factor V activity during whole blood clotting is dependent upon the ability of circulating inhibitors to inactivate the released protease. The most potent of these is the serpin, α1-proteinase inhibitor. When neutrophils undergo activation, oxygen metabolites are simultaneously released into the local environment with the elastase. These radicals serve to oxidize and inactivate the active site of the serpin. It has been estimated that 1.25 x 107 neutrophils have the potential to inactivate 162.5 to 325 μg of α1-proteinase inhibitor via oxidative attack. Thus, in inflammatory reactions the elastase released into the microenvironment could be protected from the inhibitory effects of α1-proteinase inhibitor and therefore act as an important modulator of the factor V activity and subsequent fibrin formation that characterize this pathological process. Furthermore, it is possible that the apparent factor V deficiency in patients with both acute and chronic myeloid leukemia could be explained by the release of elastase under conditions of marked leukocytosis.

Although we have concentrated our studies on the effects of neutrophil elastase on factor V, our initial observations suggested that a number of neutrophil enzymes were capable of cleaving factor V. It would therefore be more appropriate...
to suggest that neutrophils modulate factor V activity by the combined effect of these enzymes. The role of the other neutrophil enzymes in the regulation of factor V activity is the subject of our current studies.

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