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CHAPTER 41

Increased Levels of Two Distinct Elastase-Like Hydrolases in Plasma During Septicemia

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Introduction

Many of the complications of septicemia are believed to be initiated by endotoxin, resulting in activation of the complement system and, through Hageman factor, in massive activation of the kinin, coagulation, and fibrinolytic systems. Furthermore, endotoxin has been shown to stimulate polymorphonuclear leukocytes to release large amounts of neutral proteinases, particularly elastase.[1,2] Activation of the plasma proteolytic systems together with the release of enzymes from the phagocytic cells may overwhelm the proteinase inhibitors, and therfore allow the proteinases to cause local tissue and organ damage.[2] In addition, granulocyte elastase may also contribute directly to "intravascular turmoil" through kinin formation,[3] activation and degradation of coagulation factor XIII,[4] as well as inactivation of antithrombin III,[5] a key coagulation system regulator.

The present experiments were undertaken to measure and characterize elastase(s) and other proteinases released into the plasma during septicemia. Earlier reports of immunreactive α_1-antitrypsin elastase complexes in serum of septic patients[1] and endotoxin-treated dogs[2] suggested that α_2M(α_2-macroglobin)-proteinase complexes would also be present under these conditions. The methods used in the present study are based on the fact that α_2M-bound proteinases retain hydrolytic activity toward small molecular weight synthetic substrates, but are unable to attack large (protein substrates.)[8]

Materials and Methods

Plasma samples were obtained from six patients who had undergone major abdominal surgery and later developed septicemia. The samples were taken at irregular intervals for approximately 48 hours following signs of generalized infection. Control plasma was obtained from six healthy donors.

Plasma proteinase levels were quantified[6] by measuring the release of p-nitroaniline from the following chromogenic sub-

strates: Succ-Ala_3-p-NA* (Bachem, Bubendorf, CH), MeO-Succ-Ala_2-Pro-Val-p-NA† (CIBA-GEIGY, Basle), substrates for elastaselike enzymes; Bz-Arg-p-NA;‡ (Sigma, St. Louis, Mo.), a substrate for trypsinlike enzymes; and Succ-Phe-p-Na§ (Sigma), a substrate for chymotrypsinlike enzymes. In the assay, 0.2 ml of plasma was diluted with 0.2 ml of 0.1 M Tris-Cl buffer, pH 7.5, containing 1 mM substrate (except for Succ-Ala_3-NA, which was 5 mM). The mixture was incubated at 37° C for 1–6 hours and the reaction was stopped by the addition of 1 ml of 7.5% trichloroacetic acid. The protein was removed by centrifugation, and the nitroaniline released was reacted to form a purple azo-dye by addition of 25 μl each of sodium nitrite (1.8%), ammonium sulfamate (9%), and N-1-naphthyl-ethylenediamine di-HCl (1.8%, Sigma). The absorbance of the colored product was measured at 550 nm. $Alpha_2$-macroglobulin was quantified according to a modification[6] of the method of Gantrot.[8]

Separation of elastaselike enzymes from plasma was accomplished by applying a 2.75-ml sample to a column (2.6 × 94 cm) of Sephacryl S-300 superfine (Pharmacia, Uppsala), previously equilibrated with 0.02 M sodium citrate buffer, pH 6.5, with 0.15 M NaCl and 0.04% NaN_3. The column was eluted with the same buffer at a flow rate of 13.5 ml/hr.

Results and Discussion

Plasma samples taken at several times within a 2-day period from six patients suffering from severe septicemia were analyzed for their content of peptide hydrolases and compared to samples taken from six normal volunteers. Plamsa from all six patients had markedly elevated levels of hydrolases cleaving the four substrates. The degree of elevation is illustrated Figure 1, where the hydrolase levels in pooled plasma from the normal individuals (N) and from the septic patients (S) are compared.

In order to examine more closely the elastase-like hydrolase activities in septic plasma, a sample with high activities was fractionated on a column of Sephacryl S-300 superfine (Fig. 2, top). The hydrolase activity of the elastaselike enzymes showed two main peaks. The First peak (Peak I) had a molecular weight $> 10^6$ daltons and cleaved the Succ-Ala_3-p-NA substrate, but was nearly devoid of MeO-Succ-Ala_2-Pro-Val-p-NA hydrolase activity. As shown in Table 1, this enzyme was completely inactivated by 1,10 phenanthroline, an inhibitor of metalloenzymes, but was insensitive to phenyl-methyl-sulfonyl-fluoride (Pms-F), a small molecular weight inhibitor of serine proteinases, as well as to Eglin C and the Bowman-Birk soybean, both inhibitors of leukocyte elastase. In addition, it was sensitive to EDTA but unaffected by the sulfhydryl reagents, dithiothreitol β-mercaptoethanol.

The second peak eluted together with α_2M (Mw $\sim 8 \times 10^5$ daltons) and hydrolyzed both the MeO-Succ-Ala_2-Pro-Val-p-NA and the Succ-Ala_3-p-NA substrates, the former being hydrolyzed about six times faster. This enzyme activity (Table 1) was insensitive to 1,10 phenanthroline, but it was sensitive toward Pms-F, as well as toward Elgin C, which has previously been shown to inhibit leukocyte elastase complexed with α_2M (M.J., unpublished observation). A significant degree of inhibition is also observed with the relatively small molecular weight Bowman-Birk soybean inhibitor.

Based on the molecular weight, substrate specificity, and inhibitor pattern, we conclude that the second peak of peptide hydrolase activity corresponds to α_2M-leukocyte elastase complex. This is further

*N-succinyl-L-alanyl-L-alanyl-L-analyl-p-nitroanilide.

†Methoxy-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valyl-p-nitroanilide.

‡N-benzoyl-L-arginyl-p-nitroanilide.

§Succinyl-phenylalanyl-p-nitroanilide.

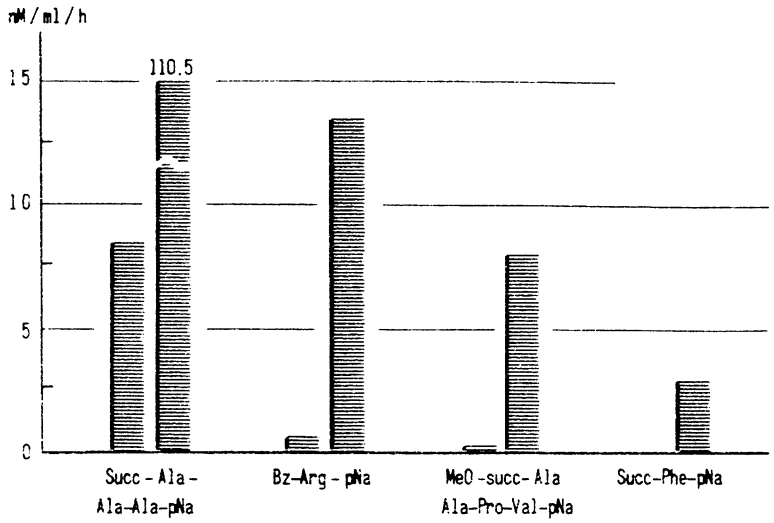

FIGURE 1. Hydrolysis of different peptide nitroanilides by normal plasma and plasma from septic shock patients (pool of six).

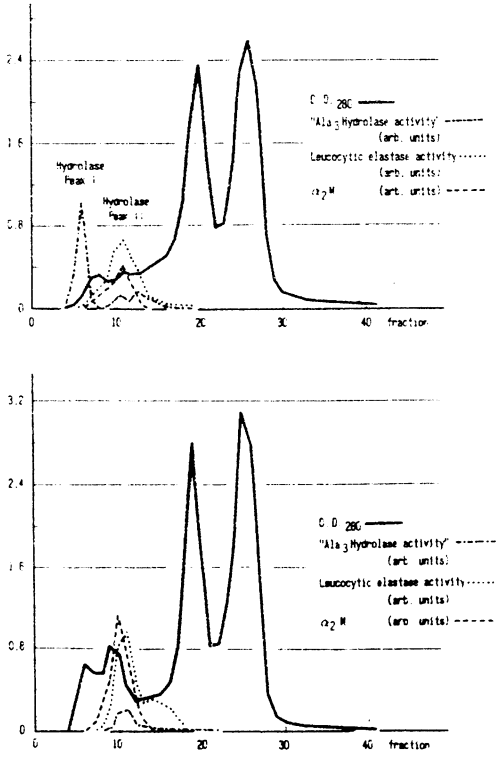

FIGURE 2. Top: separation of plasma proteins from a septic shock patient on Sephacryl 5300. Bottom: calibration of Sephacryl 5300 with normal human plasma to which purified human leukocyte elastase was added.

supported by another experiment, where isolated purified leukocyte elastase was added to normal plasma and fractionated on the same Sephacryl column: the artificial α_2M-elastase complexes eluted at the same place (Fig. 2, bottom) and showed an identical inhibition pattern. Peak I activity on the other hand is clearly unrelated to leukocyte elastase; it is a metalloenzyme, very highly specific for Succ-Ala_2-p-NA, and has a molecular weight which is so large as to suggest aggregates or association with small particles. Similar metal-dependent peptide hydrolase activities have been described previously in patients with liver disease.[9,10]

Septicemia and septic shock is apparently associated with a raise in serum leukocyte elastase levels, shown here as α_2M complexes and in agreement with the occurence of α_1-AT complexes demonstrable by immunological means.[1,2,12] In addition, there occurs a massive increase of a metalloenzyme hydrolyzing Succ-Ala_3-p-NA. This latter activity may reflect an involvement of the liver—a general feature of endotoxemia and septic shock.[11]

TABLE 1. Inhibition of partially purified peptide hydrolases from plasma of spetic shock patients

Inhibitors		Substrate	
		Peak I: Succ-Ala-Ala-Ala-pNA	Peak II: MeO-succ-Ala-Ala-Pro-Val-pNA
1,10 Phenanthrolin	1 mM	96%	2%
Phenylmethyl-sulfonyl fluorid	1 mM	7%	91%
Eglin	10 μg/ml	1%	96%
Bowman-Birk soybean inhibitor	10 μg/ml	0%	44%
Bowman-Birk soybean inhibitor	50 μg/ml	14%	75%

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