

HANDBOOK *of* MEDIATORS *in* SEPTIC SHOCK

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PHAGOCYTE PROTEINASES IN MULTIPLE TRAUMA AND SEPSIS: PATHOMECHANISMS AND RELATED THERAPEUTIC APPROACHES*

M. Jochum, W. Machleidt, and H. Fritz

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* This chapter is an extended version of the original contribution: Jochum M., Machleidt W., Fritz H., Proteolysis-induced pathomechanisms in acute inflammation and related therapeutic approaches, in *Molecular Aspects of Inflammation*. 42. Colloquium Mosbach 1991. Sies H., Flohé L., Zimmer G., Eds., Springer-Verlag, Berlin, 1991. 73. Reproduction is kindly permitted by Springer-Verlag.

I. INTRODUCTION

A. ASPECTS OF PROTEOLYTIC PATHOMECHANISMS IN INFLAMMATION

There are a multitude of inflammatory mediators and reactions investigated hitherto and claimed as being at least contributory to the development of an inflammatory process. Proteolysis-induced pathomechanisms seem to play a major role in the primary response to and the ultimate outcome of the organism to inflammatory stimuli such as tissue destruction due to multiple trauma and major surgery or invasive microbes and endotoxins in sepsis. Independent of the etiology of the insult, the inflammation response is primarily directed towards inactivating and eliminating the deleterious agents and to initiate the process of repair and healing. Yet, the activation of the complex interacting cellular and humoral defense mechanisms necessary for this purpose also carries with it the risk of damaging healthy tissue, thus perpetuating the inflammatory process. In this respect, the lysosomal serine proteinase elastase and the cysteine proteinase cathepsin B of the primary inflammatory cells, polymorphonuclear (PMN) granulocytes and monocytes/macrophages, respectively, are supposed to be potent effectors of proteolytic tissue damage if they are discharged extracellularly in high amounts during activation and disintegration of the phagocytes. Moreover, proteinases of humoral origin (plasma kallikrein, thrombin, plasmin, complement esterases) and protein split products (permeability increasing fibrinopeptides and fibrin monomers, fibronectin peptides, complement-derived anaphylactic factors, such as C3a, C4a, and C5a, vasoactive kinins, arachidonic acid metabolites, etc.) generated by their proteolytic action have been proven to be of major importance as strong stimulators of the primary defense cells.

In addition to lysosomal proteinases, highly reactive oxygen species produced by the respiratory burst are also extracellularly liberated during phagocytosis. This means that vital structural elements (basal membranes, cell receptors, fibronectin, elastin, collagens, proteoglycans, etc.) as well as humoral factors including a wide variety of plasma proteins (review of literature in Jochum¹ and Machovich and Owen²) in close vicinity to the phagocytizing cells may be impaired unless the lysosomal proteolytic enzymes and oxidants are inactivated by their physiological regulators, the proteinase inhibitors (α_1 -proteinase inhibitor, α_2 -macroglobulin, cysteine proteinase inhibitors) and antioxidants (superoxide dismutase, catalase, glutathione redox system, ceruloplasmin).

Yet, due to an overstressed phagocytic activity of PMN granulocytes and monocytes/macrophages during severe inflammation, the main antagonist of PMN elastase, the α_1 -proteinase inhibitor (α_1 PI), is highly susceptible not only to proteolytic cleavage by cysteine and metallo proteinases released from the mononuclear cells, but also to oxidative denaturation of the reactive inhibitory site in the molecule.³ This enables an unrestricted local digestive activity by PMN elastase combined with fatal consequences for the hemostasis system. As shown by several authors (reviewed in References 1 and 2), PMN elastase can easily degrade and inactivate the principal inhibitors (antithrombin III, α_2 -plasmin inhibitor, plasminogen activator inhibitor 1, C1-inactivator) of proteinases of the blood cascade systems (clotting, fibrinolysis, complement), thus allowing the maintenance of life-threatening consumption of hemostasis factors and the additional production of potent stimulators of the phagocytes as mentioned above. Moreover, the proteolytic inactivation of the blood cascade inhibitors may be greatly enhanced by their oxidative denaturation in the surroundings of phagocytizing cells.⁴

Just recently the proteolytic degradation of the main human cysteine proteinase antagonists, cystatin C and kininogen, by PMN elastase has also been demonstrated,^{5,6} which may further aggravate the destructive potency of monocyte/macrophage-derived cysteine proteinases which are extracellularly discharged due to serious incidents such as multiple trauma and sepsis.

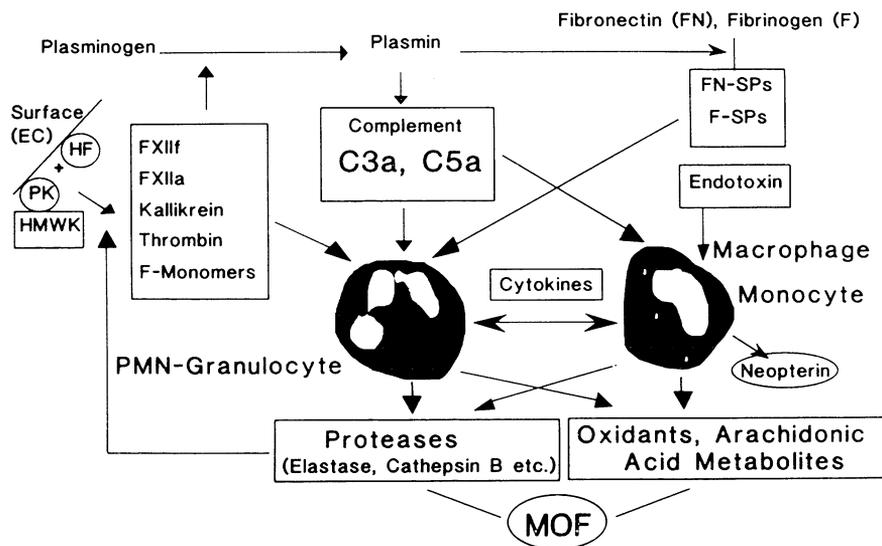


FIGURE 1. Schematic depiction of humoral and cellular proteolytic pathomechanisms involved in the development of multiple organ failure (MOF). Destructive proteases (elastase, cathepsin B) and other cell constituents are released after activation of the phagocytes by blood cascade proteinases (plasma kallikrein, factor XIIa, thrombin, etc.) and polypeptides (fibrinogen and fibronectin split products, complement factors C3a and C5a, etc.). For detailed explanations see text.

The tremendously complex interrelationship of cellular and humoral proteolytic pathomechanisms eventually contributing to the development of multiple organ failure (MOF) in the course of severe acute inflammation is depicted in a very simplified version in Figure 1. A comprehensive review of literature on this topic is given by Jochum,¹ Jochum and Fritz,^{7,8} and Assfalg-Machleidt et al.⁹

B. RATIONALE FOR THERAPEUTIC USE OF PROTEINASE INHIBITORS

To obtain fundamental information about the extent to which proteinases may participate in the initiation and perpetuation of inflammatory processes, the following indications — as a modification of the Koch-Dale criteria — have to be proven in clinical and/or experimental studies:

1. The release of the lysosomal proteinases (e.g., PMN elastase, cathepsin B) and the activation of proteolytic blood cascade enzymes has to be verified in correlation to the severity of the inflammation.
2. The consumption of proteinase inhibitors and other plasma factors susceptible to proteolytic degradation should coincide with the occurrence of proteolytic activity.
3. Specific split products of functional proteins generated by the proteolytic action of lysosomal proteinases have to be shown in correlation with the extracellular release of these enzymes.
4. Administration of exogenous phagocyte proteinases in animals should elicit reactions similar to those observed during acute inflammation.
5. The use of specific exogenous proteinase inhibitors as therapeutic tools should prevent or at least reduce to some extent severe signs of inflammation.

In the following part of this chapter, characteristic results on these demands primarily obtained from our own clinical and experimental studies dealing with multiple trauma and/

or septicemia are given in more detail. Using the same specific test systems, these findings and those of other authors, which will be also briefly mentioned, demonstrate clearly the involvement of proteolysis-induced pathomechanisms in acute inflammation regardless of the inciting cause. Therefore, proteinase inhibition may be an indispensable and therapeutic approach to reduce the incident of organ failure and lethality in such disease states in the future.

II. METHODOLOGY

Serially drawn citrated plasma, bronchoalveolar lavage fluid (BALF), and peritonitis exudate samples were used to quantify proteinases, inhibitors, and plasma proteins as well as their split products.

Measurements of the lysosomal serine proteinase PMN elastase were carried out with a commercially available, highly specific two-site sandwich enzyme immunoassay kit (E. Merck, Darmstadt, Germany), which detects elastase only as an inactive complex with α_1 PI (detailed description in Neumann and Jochum¹⁰). The normal range of circulating complexed elastase in healthy people due to the physiological turnover of PMN cells is between 60 and 120 ng/ml without the proteolytically active enzyme being detectable. Since, in contrast to plasma, BALF and peritonitis exudate samples may contain active elastase in addition to the complex, parts of these specimens were also incubated with a surplus of α_1 PI *in vitro* and reassayed for an increase in elastase — α_1 PI complex as a measure of enzymatic elastase activity *in vivo*.

The cysteine proteinase cathepsin B activity (upper normal plasma level: 50 mU/l) was quantified using a specific fluorometric peptide substrate as described by Assfalg-Machleidt et al.⁹ Caseolytic activity of local body fluids due to active elastase and/or cathepsin B was detected using resorufin-labeled casein (Boehringer, Mannheim, Germany) as a substrate. The method is outlined in detail by the manufacturer (Boehringer).

The coagulation proteinases plasma kallikrein and thrombin were estimated by their cleavage activity on the chromogenic peptide substrates S-2302 (Kabi, Stockholm, Sweden) and Chromozym TH (Boehringer, Mannheim, Germany), respectively, after the turnover of the proenzymes to the active proteinases.

The inhibitory activities of antithrombin III (AT III) and α_1 PI were measured with commercially available test systems (Coatest Antithrombin, Kabi, Stockholm, Sweden; α_1 -Antitrypsin Farbstest, Boehringer, Mannheim, Germany) applying chromogenic peptide substrates for thrombin (S-2238) and trypsin (BAPA), respectively. The antigen levels of the inhibitors were quantified with commercial radial immunodiffusion plates (NOR-Partigen plates, Behringwerke, Marburg, Germany).

Opsonic activity, antigen levels and split products of the opsonins IgG and complement factor C3 were determined as outlined in Billing et al.¹¹⁻¹³ and Machleidt et al.¹⁴

An elastase-specific split product of the A α -chain of fibrinogen, called fibrino-elastase-peptide (FEP; equivalent to the A α 1-21 peptide described by Weitz et al.¹⁵), was quantified with a two-step competitive immunoassay just recently developed in our laboratory by Gippner-Steppert.¹⁶

Clinical methods, criteria for grading the severity of the diseases, treatment of patients, and experimental therapeutic studies are extensively described in the original publications cited in the following sections.

III. RESULTS AND DISCUSSION

A. PROTEOLYSIS-INDUCED PATHOMECHANISMS IN HUMANS

1. Multiple Trauma and Infection

In an early preliminary clinical attempt including 27 polytraumatized patients, we were able to show that severe injuries of at least two body regions (thorax, abdomen, skeletal system) induced a prompt and, more or less, long-lasting release of PMN elastase into the circulation of the patients (Dittmer et al.¹⁷). Measurements were carried out every 4 h up to the 28th h and thereafter every 6 h up to the 52nd posttraumatic hour. The maximum increase between five- and 30-fold of normal PMN elastase plasma levels correlated well with the clinical injury severity score within 8 to 12 h after the traumatic event. Yet, due to logistic limitations of the study, it was not possible to evaluate a predictive value of the extracellularly discharged PMN elastase for the development and outcome of trauma-induced (multiple) organ failure.

In a second approach concerning 24 multiply injured patients, we could demonstrate that the primary activation of PMN granulocytes, immediately after the polytraumatic event, is followed by repetitive increases of complexed elastase in plasma in those patients who developed ARDS (adult respiratory distress syndrome) and additional organ failure (Jochum et al.¹⁸). This multiple organ insufficiency in our patients was mainly due to septic complications. In agreement with findings of Nuytinck et al.¹⁹ and Redl et al.²⁰ elevated plasma levels of elastase correlated well with the severity of injuries and the occurrence of MOF. Moreover, they discriminated to a reasonable degree at an early stage in the clinical course between later survival or mortality.

To clarify further the potential role elastase — and additionally cathepsin B — may play in progressive posttraumatic complications, we recently accomplished a more extended exploration. One hundred severely injured patients, fulfilling previously defined entry criteria, have been enrolled in a prospective study (directed by Drs. Nast-Kolb and Waydhas, Surgical Clinic City, University of Munich) on inflammatory mediators and MOF associated with polytraumatic events (Nast-Kolb et al.,²¹ Waydhas et al.²²). The collection of blood samples and recording of clinical data were started within 30 min after arrival of the patients in the emergency room (about 1 h after the accident) and continued on a 6-h basis up to 48 h. Subsequently, the monitoring interval was extended to 24 h for a period of 14 d. Thereafter, the clinical course was recorded until either transfer to a general ward or death of the patient.

Retrospectively, the patients could be assigned to three different groups: 16 out of them died due to MOF between 3 and 28 d (mean survival time: 16 d) after the traumatic incident (group I), 47 patients survived the development of organ failure (group II), and 37 patients overcame the accident without evident signs of organ dysfunctions (group III).

The extracellular release of PMN elastase and monocyte/macrophage-derived cathepsin B into plasma of the patients within the three outcome groups is depicted in Figure 2. PMN elastase was elevated in all groups significantly above normal (upper range 120 ng/ml) within 1 to 2 h after trauma and showed an additional increase up to the 12th posttraumatic hour. The differences between mean plasma levels in patients with and without organ failure (groups 1 and 2, and group 3, respectively) were highly significant ($p < 0.01$) throughout the entire observation period. Moreover, patients dying due to organ failure (group I) and those who survived organ dysfunctions (group II) could be significantly ($p < 0.05$) differentiated according to their mean PMN elastase plasma concentrations from the third post-

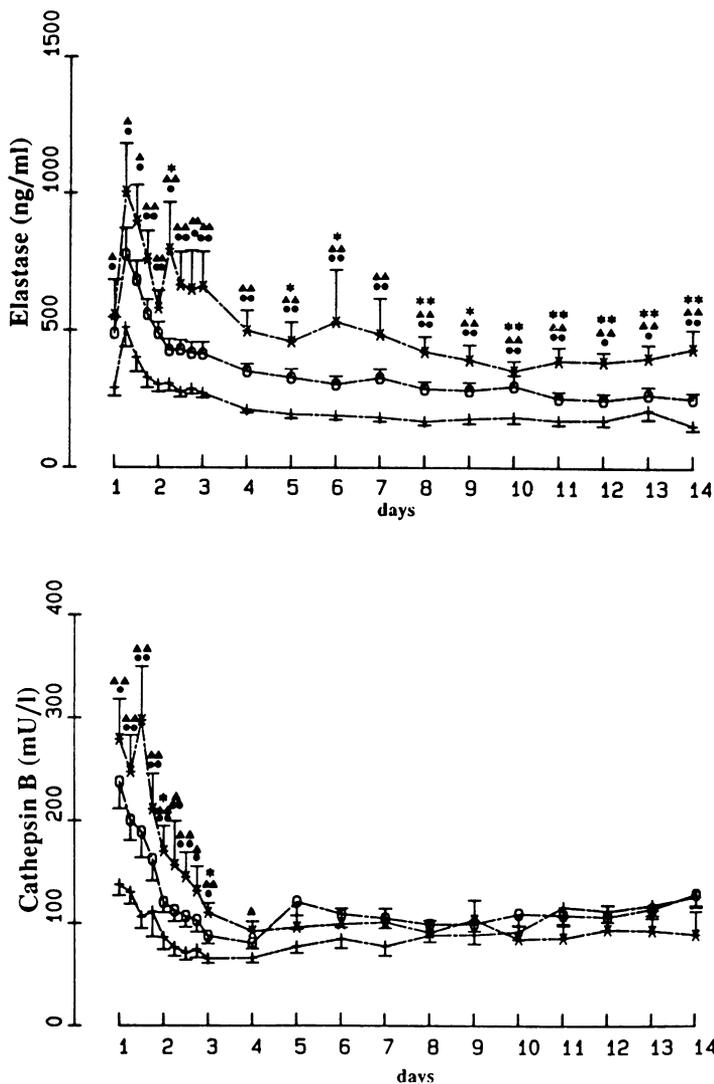


FIGURE 2. Mean values (\pm SEM) of polymorphonuclear elastase (in complex with α_1 -proteinase inhibitor) and cathepsin B in plasma of multiply injured patients assigned to three outcome groups: Group I ($n = 16$), nonsurvivors with multiple organ failure (*—*); group II ($n = 47$), survivors with reversible organ failure (○—○); group III ($n = 37$), survivors without organ failure (+—+). (*) $p < 0.05$, (**) $p < 0.01$ for group I vs. group II; (●) $p < 0.05$, (●●) $p < 0.01$ for group I vs. group III; (▲) $p < 0.05$, (▲▲) $p < 0.01$ for group II vs. group III.

traumatic day onward. Cathepsin B plasma levels reached their maxima at least 6 h before complexed PMN elastase (Figure 2). This early cathepsin B activity in circulating blood proved to be a sensitive and specific predictor of subsequent developing organ failure. Yet, it did not discriminate between lethal (group I) and reversible (group II) dysfunctions at that time. In contrast to PMN elastase, cathepsin B activity returned to only slightly elevated levels within 3 d in all patients (Figure 2), thus, not allowing a further differentiation of the three outcome groups. From these data, the conclusion can be drawn that the early release of lysosomal phagocyte proteinases, after a severe traumatic event, contributes to the occurrence of forthcoming organ failure, and that at least the ongoing discharge of PMN elastase may be involved in the exacerbation of the clinical situation. The latter seems to

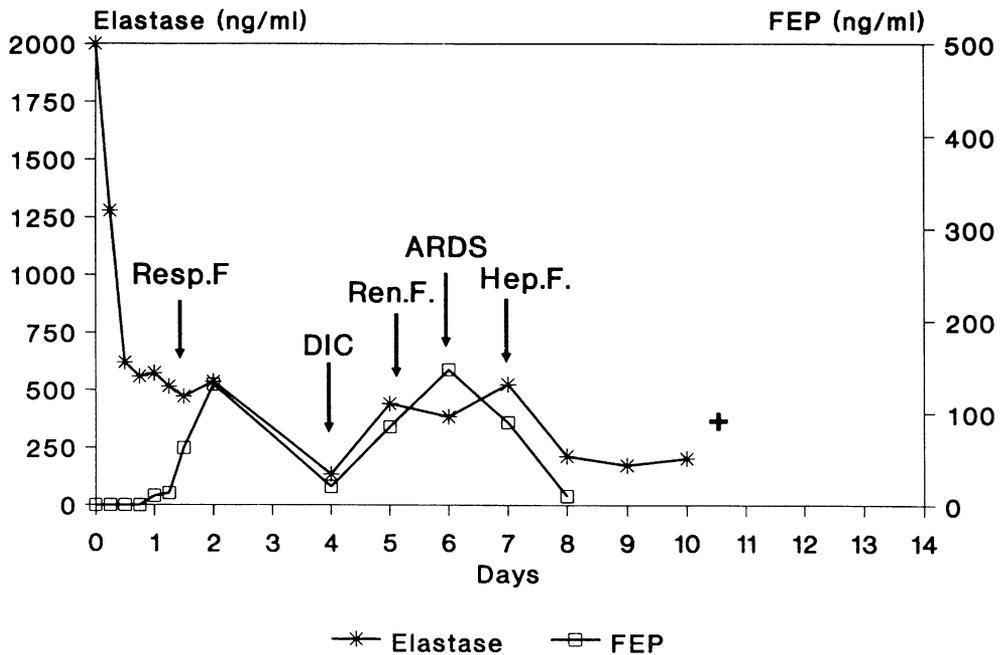


FIGURE 3. Plasma levels of polymorphonuclear elastase (in complex with α_1 -proteinase inhibitor) and fibrino-elastase-peptide (FEP) in a polytraumatized patient with lethal multiple organ failure. (Resp.F.) respiratory failure; (DIC) disseminated intravascular coagulation; (Ren. F.) renal failure; (ARDS) acute respiratory distress syndrome; (Hep.F.) hepatic failure.

be confirmed by the appearance of the elastase-induced split product FEP of fibrinogen in correlation to the continuous release of elastase and the development of lethal MOF as outlined in Figure 3 for the first time for a severely injured patient.

Since blunt trauma as well as bacterial complications and sepsis initiate the release of phagocyte proteinases,^{7,19,20,23} we compared the plasma level patterns of PMN elastase and cathepsin B in subgroups of trauma patients suffering from infection/sepsis and those who did not develop these complications.²² In about 70% of patients with organ failure, early dysfunctions (usually respiratory insufficiency) became obvious during the first two post-traumatic days. Late organ failure, predominantly due to liver failure, emerged after the third day posttrauma, whereas MOF was diagnosed in 32% of all group I and II patients between day six and eight. Infections appeared around the third day post trauma and remained fairly constant for about 1 week. Bacterial sepsis occurred slightly delayed. According to the three outcome groups infection/sepsis was verified for 81%/50% of the patients in group I, for 74%/36% in group II, and for 24%/5% in group III, respectively. Summarizing the clinical data, it turned out that in 97% of the patients with an early onset of organ failure, infection started at least 2 d later than the organ insufficiencies. In only half of those patients, infection or sepsis led to a consecutive deterioration of the clinical situation. In contrast, in 50% of the 18 patients with a late onset of organ failure, infection and sepsis preceded the disturbance of organ functions and seemed to have pathogenic significance. Interestingly, only PMN elastase showed an obvious correlation with infection or sepsis. Since significantly higher plasma levels were measurable even before onset of these entities compared to a similar posttraumatic course without such complications (shown in Figure 4 for group II patients), the conclusion can be drawn that the granulocytic proteinase may facilitate and maintain the occurrence of posttraumatic infections and sepsis. This assumption is also confirmed by recently published data on progressive organ failure in a prospectively studied

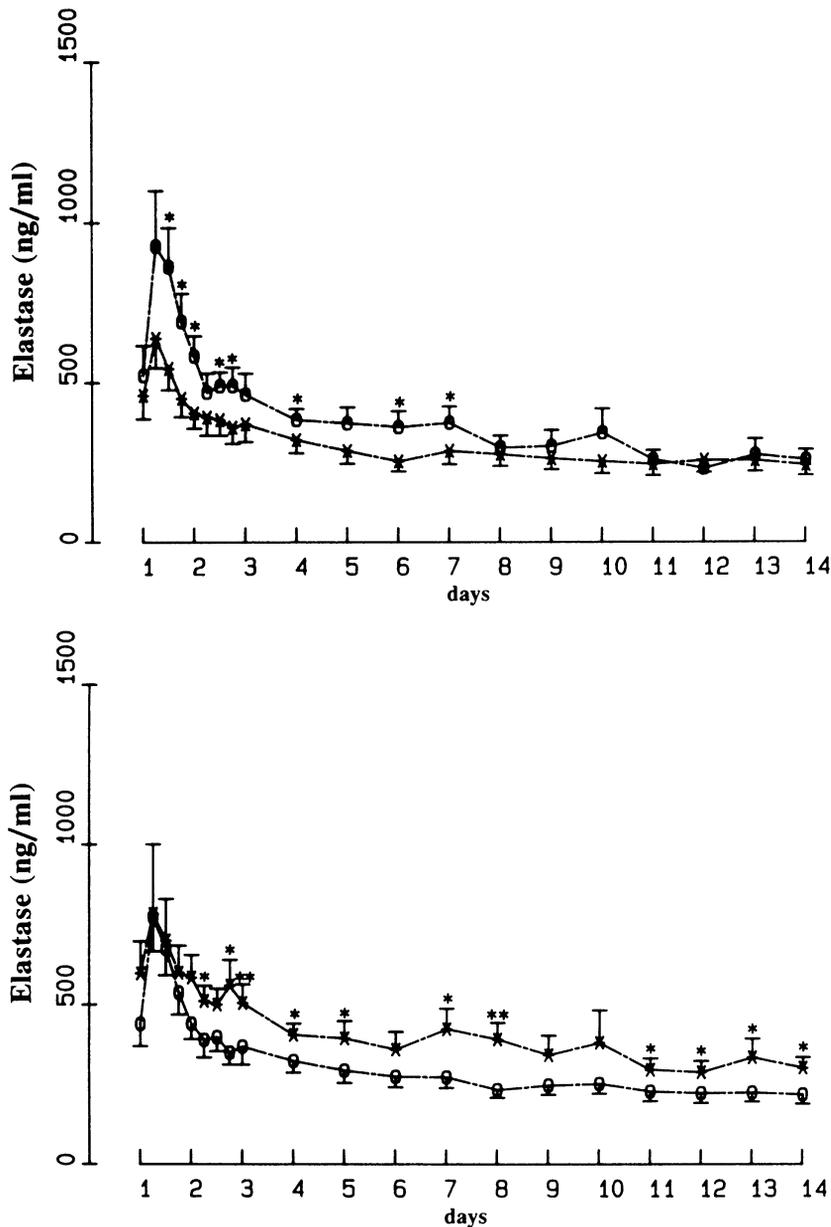


FIGURE 4. Mean values (\pm SEM) of PMN elastase (in complex with α_1 -proteinase inhibitor) in plasma of trauma patients with reversible organ failure (group II). (a) Patients with infection (*—*; $n = 35$) and without infection (○—○; $n = 12$) — (*) $p < 0.05$ infection vs. no infection. (b) Patients with sepsis (*—*; $n = 17$) and without sepsis (○—○; $n = 30$) — (*) $p < 0.05$, (**) $p < 0.01$ sepsis vs. no sepsis.

group of well-defined, multiply injured patients.²⁴ Although no information is given about the time of onset of the septic state, significantly higher PMN elastase plasma levels were presented for 18 septic, in comparison to 17 nonseptic patients from the 24th h up to the 8th d posttrauma.

Interestingly, the main clotting inhibitor, AT III, showed a significantly lower inhibitory activity ($p < 0.01$) in plasma of group I and II patients vs. those of group III during the whole observation period and between nonsurvivors (group I) vs. survivors with organ failure

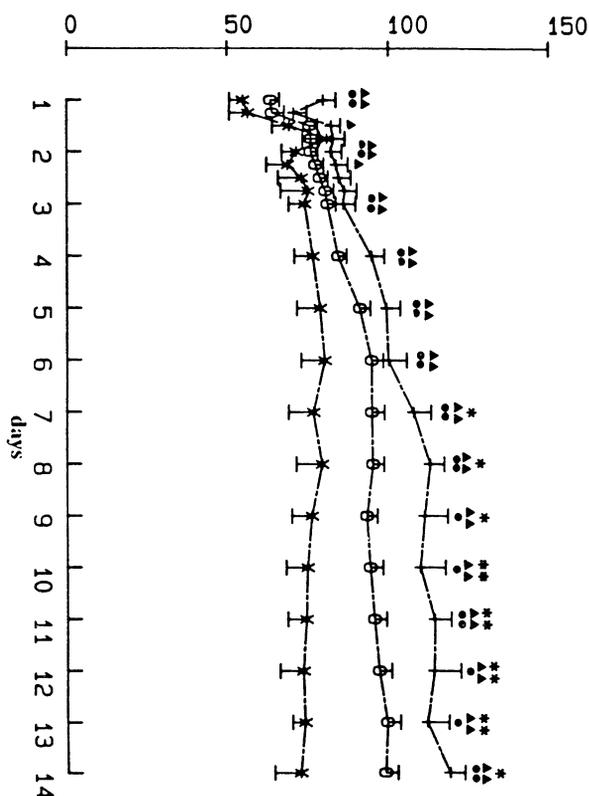


FIGURE 5. Mean values (\pm SEM) of antithrombin III inhibitory activity in plasma of multiply injured patients assigned to three outcome groups. For legend see Figure 2.

(group II) beginning with the first posttraumatic week onwards (Figure 5). Similar results were obtained concerning the rapid turnover of the coagulation proenzymes prekallikrein and prothrombin to kallikrein and thrombin, the latter being the essential target proteinase of AT III. Thus, plasma levels of AT III, prekallikrein, and prothrombin, which were 60% below normal in the early posttraumatic phase, were highly associated with the later appearance of severe organ dysfunctions, indicating that in addition to the release of lysosomal proteinases, an overwhelming activation of the humoral proteolytic cascade systems is also conducive to the perpetuation of the posttraumatic inflammatory process. Yet, neither levels of AT III nor those of the clotting proenzymes differentiated between septic and nonseptic patients in any of the three outcome groups.²² The highly predictive value of early, low AT III inhibitory activities for a forthcoming exacerbation of posttraumatic organ dysfunctions eventually leading to ARDS and MOF was also emphasized by Schramm and Spannagl²⁵ reporting on 57 prospectively studied trauma patients.

In this clinical study on multiply injured patients (a detailed study protocol is given by Sturm²⁶), we have been especially interested in local proteolysis-induced mechanisms, which might be involved in the pathogenesis of the most severe lung dysfunction, ARDS. Daily drawn BALF samples (method described by Obertacke et al.²⁷) allowed us to confirm a significantly increased local discharge of the phagocyte proteinases, elastase, and cathepsin B, in subjects at high risk to develop ARDS (Jochum²⁸). Although the α_1 PI antigen levels in all BALF specimens of the traumatized patients were far above the normal values of healthy volunteers, these amounts were apparently not sufficient in most cases to completely inhibit the PMN elastase released in the local epithelial milieu of the alveoli despite an up-

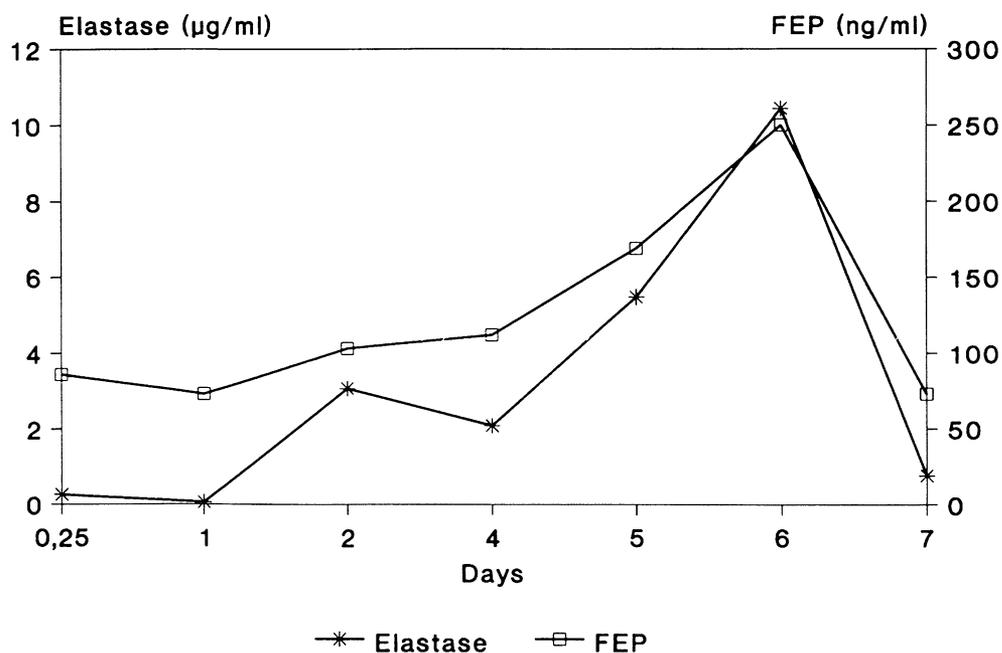


FIGURE 6. Proteolytic activity (degradation of resorufin-casein with and without addition of α_1 -proteinase inhibitor) and free PMN elastase in serial bronchoalveolar lavage fluids of a severely traumatized patient. Acute respiratory distress syndrome and multiple organ failure persisted from the 5th to the 12th posttraumatic day.

to 40-fold molar surplus of the inhibitor over the enzyme. Therefore, the obviously deficient inhibitory capacity of α_1 PI may have been caused by proteolytic and oxidative denaturation as well. Applying special assay systems, both ways of α_1 PI destruction were demonstrated by Schraufstatter et al.²⁹ at least in some individual BALF samples of patients with manifest ARDS. Although other authors could not detect elastase activity against high molecular weight protein substrates in BALF samples of ARDS patients (Idell et al.³⁰ Wewers et al.³¹), we were recently able to show caseinolytic activity in a variety of BALF samples (kindly given to us by Dr. Obertacke, Surgical Clinic, University of Essen) from trauma patients with severe lung dysfunctions (Machleidt et al.¹⁴). *In vitro* incubation of these specimens with α_1 PI nearly completely abolished this proteolytic activity (Figure 6) and gave rise to an additional increase of the elastase- α_1 PI complex, indicating that the enzymatic activity present in the alveolar environment was mostly due to PMN elastase.

Moreover, the destructive potency of elastase *in vivo* could be directly documented for the first time by the proof of augmented generation of FEP in close correlation to the rising amount of the extracellularly discharged PMN enzyme in BALF samples of patients with aggravating respiratory failure (figure 7). Hence, the aggressive components of inflammatory cells, especially the lysosomal proteinases, may indeed contribute to the occurrence of lung dysfunctions in severely traumatized patients.

2. Sepsis and Peritonitis

In several prospective clinical studies on more than 200 patients suffering from bacterial infections after major surgical treatments, we could demonstrate an increasing release of PMN elastase into the circulation in accordance with the worsening of the inflammatory reaction (Duswald et al.³² Inthorn and Jochum;³³ Jochum et al.²³). Patients without postoperative infections showed only moderate transiently elevated plasma levels of complexed elastase (up to three times that of normal) after the operation, whereas onset and course of

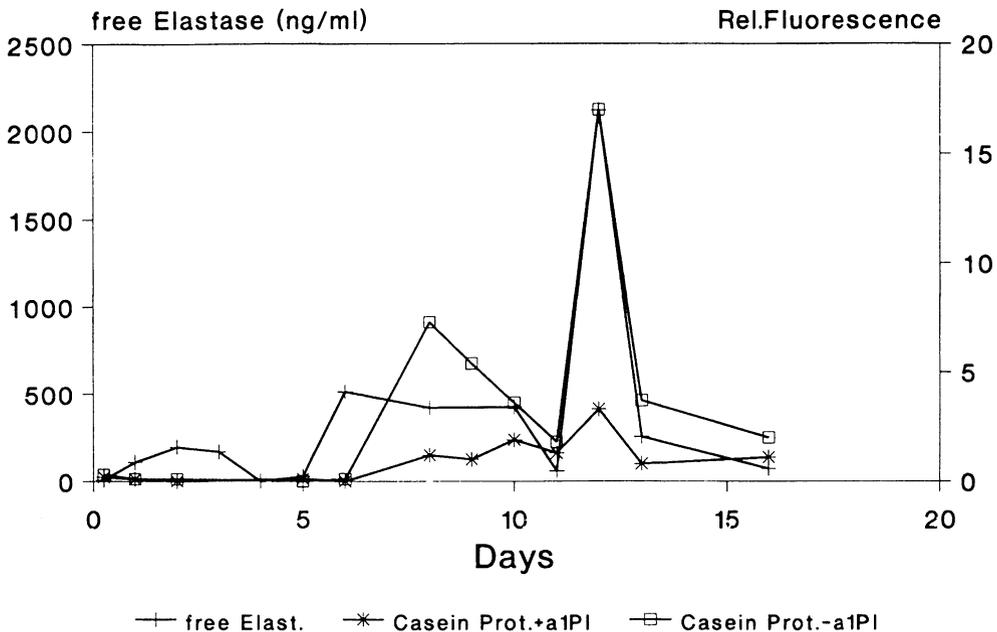


FIGURE 7. Total amount of PMN elastase (measured as complex with α_1 -proteinase inhibitor) and fibrin-elastase-peptide (FEP) in serial bronchoalveolar lavage fluids of a severely traumatized patient with reversible respiratory dysfunction from the second to the seventh posttraumatic day.

sepsis were characterized by markedly increased concentrations up to 30-fold in individual cases. As a mean, in patients with persisting septicemia the elastase plasma levels remained significantly elevated until death, while a recovery phase was accompanied by an obvious return to normal values. A detailed evaluation of our data^{1,33} exhibited that in eventually dying patients a significant increase of complexed elastase occurred 2 to 3 d before clinical manifestation of severe sepsis, followed by a transient decrease and an additionally significant rise, when sepsis was clinically diagnosed (figure 8). In surviving patients, however, the tremendous increase of PMN elastase in plasma did coincide with the clinical manifestation of the severe infection, whereas the recovery phase was clearly indicated earlier by declining elastase levels compared to the clinical assessment (Figure 8). Thus, measuring PMN elastase in closely matched consecutive plasma samples (taken every 6 h) of severely ill patients did not only allow an early diagnosis of the clinical state, but also suggested an involvement of the granulocytic proteinase in the development of severe infection and sepsis.

When complexed elastase increased in plasma, a prominent reduction of the humoral blood cascade proenzymes, prekallikrein and prothrombin, as well as of the AT III inhibitory activity, was measurable. This indicates that the proteolytic mechanisms in this pathological situation are quite similar to those seen in the course of developing posttraumatic organ failure. In addition, we could demonstrate in some pilot measurements that during a septic shock phase the monocyte/macrophage-derived cathepsin B was released in a manner similar to the PMN elastase, thus intensifying the extracellular proteolytic capacity.²³

Just recently we were able to verify such a destructive proteolytic potency in a still ongoing prospective study (clinically directed by Dr. Inthorn, Surgical Clinic Großhadern, University of Munich) on septic patients. To be enrolled in this investigation, patients had to fulfill all four previously defined septic criteria (septic focus and/or positive blood culture; body temperature $>38.5^{\circ}\text{C}$; leukocytes $>15,000/\text{l}$ or $<5,000/\text{l}$; platelets $<100,000/\text{l}$ or drop of platelets $>20\%$ within 24 h ensuring a severe inflammatory situation. In patients with lethal outcome, the generation of the elastase-induced fibrinogen split product FEP was well

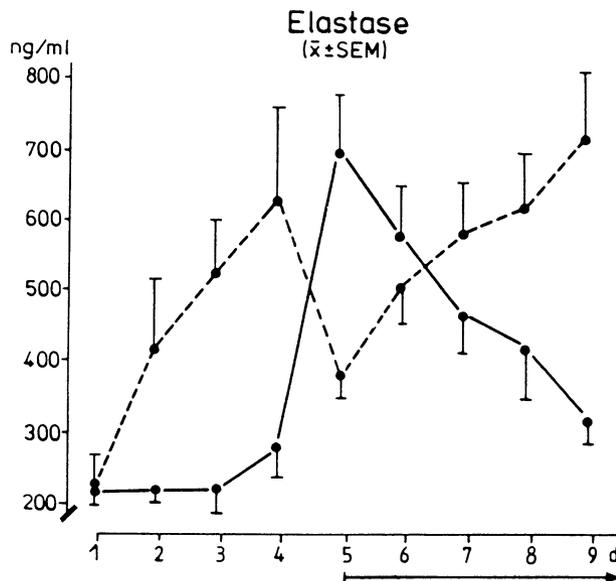


FIGURE 8. Plasma levels of PMN elastase (in complex with α_1 -proteinase inhibitor) in patients developing severe infection/sepsis. Days 1 to 4: clinical course with minor or without complications; days 5 to 9: clinically diagnosed life-threatening infection/sepsis. (-----) nonsurvivors, $n = 11$; (—) survivors, $n = 11$.

correlated to high plasma levels of PMN elastase. Moreover, the unspecific proteolytic degradation of AT III, which is also susceptible to PMN elastase cleavage (Jochum et al.,³⁴ Jordan et al.³⁵), could be pointed out by an indirect method comparing the amount of AT III antigen with its inhibitory activity. In patients suffering from eventually lethal sepsis, AT III was not only considerably consumed (to about 50% of normal) throughout the whole sepsis period. The clearly lower inhibitory activity (between 20 and 40%), however, indicated also that part of the antigen level may be due either to inactive complexes of the inhibitor with clotting enzymes or to proteolytically degraded molecules. Since the latter have an only slightly lower molecular weight and nearly the same half-life as the intact molecules (Jordan et al.³⁵), they cannot be distinguished from the native AT III if quantified by radial immunodiffusion. In our patients, however, we could definitely rule out the possibility that AT III-proteinase complexes significantly contribute to the higher antigen levels in comparison to the inhibitory activity; due to their very short half-life of only a few minutes, concentrations of AT III-thrombin complexes ran up at most to only 0.01% of the overall AT III amount. Thus, elastase-inactivated AT III molecules should be present with high probability in the pathological situation of severe sepsis. This assumption is further supported by results of Jordan et al.³⁵ which demonstrate that even catalytic amounts of heparin react with elastase in a way considerably accelerating the rate of AT III inactivation by this enzyme. Since heparin exists as a minor component on vascular endothelial cells as well as a therapeutic drug in our patients, the positive regulation of hemostasis by heparin may be significantly counteracted by the adverse effect on AT III in case of a high release of elastase from PMN granulocytes adhering to the endothelial layer of the blood vessel walls. Moreover, as recently shown by Frommherz et al.,³⁶ inhibition of elastase by α_1 PI is significantly retarded in the presence of heparin, which would further allow proteolytic degradation of susceptible plasma proteins.

Results confirming our findings with respect to the release of PMN elastase and the consumption of hemostatic proteins in more than 150 patients developing infection and sepsis have been extensively published by the research group of R. Egbring (review of literature

in Egbring et al.³⁷ and Seitz et al.³⁸). The authors found not only significantly elevated elastase levels in septic patients, but also in subjects with cardiac shock and fulminant hepatic failure. Yet, the highest values (up to 50-fold that of normal) were measured in septic shock patients.

In addition, Pacher et al.³⁹ reported on a study with daily measurements of the complexed elastase in plasma of a heterogeneous group of patients which could be divided into 32 septic and 24 uncomplicated postoperative and nonpostoperative cases. In these patients a significant difference in elastase plasma levels was seen between septic nonsurvivors and nonseptic patients, whereas septic survivors showed equally high levels as nonseptic postoperative patients. Moreover, septic pulmonary insufficiency could be predicted 1 d before the need of mechanical ventilation by elastase levels above fourfold that of normal with a specificity of 80% and a sensitivity of 81%. With an even higher specificity (83%) and sensitivity (88%), this threshold level predicted MOF 1 d before an MOF-score of >5 was evaluated. These results can be taken as a further hint to the pathogenic potency of PMN elastase in the development of sepsis and MOF regardless of the inciting cause.

The predictive validity of PMN elastase plasma levels for a forthcoming postoperative infection risk was also confirmed by Fink et al.⁴⁰ In 12 of 25 patients with colon-related surgical operations the complexed elastase in plasma was still significantly elevated above normal at the fifth postoperative day, thereby indicating a forthcoming infection at least 3 d before the clinical verification of the complication on day 8 to 10. In uncomplicated cases, a steady decrease after the operation-induced elevation was noticed. In a second study of this research group⁴¹ on 56 intensive care patients undergoing abdominal surgery, the operation procedure induced a threefold increase of elastase plasma levels in 31 patients with an uncomplicated postoperative course, whereas in patients with an early postoperative sepsis, a more than fivefold elevation above normal was measured. Ten patients who survived the septic episode were characterized by a steady decrease of complexed elastase and could not be differentiated from those without sepsis from the 48th postoperative hour onwards. Septic nonsurvivors ($n = 15$), however, showed constantly high or even additionally increasing postoperative elastase levels. Thus, the authors concluded that a long lasting elevation of PMN elastase is highly indicative of a serious deterioration of the septic situation.

Just recently, Tanaka et al.⁴² compared daily measured elastase plasma levels of 16 patients with septic shock complicated by MOF with those of 30 patients with hemorrhagic shock secondary to severe injury over a period of 1 week. Whereas in the septic patients the shock period lasted for more than 7 d, patients suffering from severe injury recovered from hemorrhagic shock within 24 h. At diagnosis of septic shock, complexed elastase was elevated up to a mean plasma level of eightfold above that of normal and remained high during the ensuing week. In hemorrhagic shock the levels were also raised up to about sixfold but quickly returned to normal at recovery. In this study the increase of complexed elastase in plasma was significantly correlated to decreases of plasmatic fibronectin and factor XIII, two proteins known to be easily susceptible to proteolytic cleavage by PMN elastase.^{1,2} Significant correlation was also found between elastase activity in BALF samples and the respiratory index of the septic shock patients, thus suggesting that at least local tissue damage in sepsis and MOF may be partly due to the destructive potency of PMN elastase.

Likewise, a highly significant positive correlation between the plasma levels of complexed elastase and the respiratory index was reported by Shimanuki et al.⁴³ in 49 patients who underwent operations of various degrees of surgical stress. As the operative intervention became more severe, the plasma levels of granulocytic elastase rose. Moreover, marked and prolonged elevation of these plasma levels paralleled postoperative pulmonary complications. Patients with panperitonitis showed mean complex plasma levels of more than sevenfold of normal before operation, whereas in noninfectious underlying diseases only slightly elevated

preoperative elastase levels were measured. Coinciding with our findings,³² the elastase complex levels in cases of panperitonitis did not increase after surgery but slightly decreased over time, probably due to the removal of the abdominal inflammatory focus enabling a continuous amelioration of the infection.

Schöffel et al.⁴⁴ studied a collective of 21 patients undergoing major abdominal surgery in whom sepsis was diagnosed during their postoperative stay in ICU (intensive care unit). A persistent elevation of complexed elastase above fourfold that of normal plasma values beyond the third postoperative day or an additional increased (five- to tenfold) closely correlated with postoperative intra-abdominal infectious complications which required re-intervention. In those patients (n = 7) sepsis was clinically diagnosed between the third and sixth postoperative day. Patients with transient postoperative septic episodes showed a gradual normalization of elevated elastase plasma levels within the first 3 to 4 d after surgery. In another study monitoring the early inflammatory response in 27 peritonitis patients, the authors emphasized the significant predictive value of elevated elastase plasma levels at the time of emergency laparotomy for the later outcome (survival/death) of the patients.⁴⁵

In addition to measurements of the complexed elastase in plasma of adults, serial quantifications of this granulocytic proteinase in 306 neonates carried out by Speer and Tegtmeier⁴⁶ have proven that this inflammatory mediator is a highly sensitive and rapidly responsive indicator of neonatal septicemia as well as a helpful tool in monitoring the course of the disease. When patients with neonatal septicemia were differentiated from those with other neonatal disorders, elevated complexed elastase levels showed a sensitivity of 95% and specificity of 81% for the septic entity. Similar results were published by Tsaka and Herkner⁴⁷ on 135 newborn infants.

Diffuse peritonitis is often the source of a systemic spreading of local infectious complications eventually leading to sepsis and MOF. In previous work, we were able to demonstrate that the impairment of opsonisation in the peritoneal cavity allows the survival of huge numbers of bacteria despite the presence of intact phagocytes (Billing et al.^{11,48,49}). The proteolytic breakdown of the main opsonins immunoglobulin (IgG and complement factor C3 in peritonitis exudates correlated well with the extracellularly released lysosomal proteinases, elastase and cathepsin B, in the local body fluids. We therefore, assumed that these enzymes are the principal cause of the deficiency in opsonic capacity. To assure this supposition, isolated IgG was incubated *in vitro* either with PMN elastase and cathepsin B or (in its isothiocyanate-labeled form = FITC IgG) with a cell-free, purulent peritonitis exudate (kindly supplied by Dr. Billing, Surgical Clinic Groshadern, University of Munich). The proteolytic degradation was followed by gel chromatography of the split products exhibiting the same type of IgG cleavage pattern under all conditions (Machleidt et al.,¹⁴ Billing et al.¹³). Using resorufin-labeled casein as a substrate, similar results were obtained substantiating proteolytic elastase activity despite the presence of up to 40-fold molar surplus of α_1 PI antigen in peritonitis exudates. These findings closely resembled the already mentioned data obtained from BALF samples in trauma patients. In addition to the *in vivo* degradation of IgG and C3, we have successfully proven the generation of FEP in peritonitis exudates of 21 patients. As depicted in Figure 9, high amounts of complexed PMN elastase coincided with highly elevated FEP in the specimen drawn before surgical treatment of the abdomen of a patient with severe peritonitis. After rinsing the peritoneal cavity with 10 l of Ringer lactate solution, both parameters decreased to approximately zero. Yet, as can be seen from the abdominal drainage fluids collected between 0 and 1 h, 1 and 2 h as well as 2 and 8 h after operation, the release of PMN elastase started again, inducing also the production of FEP (Figure 9). These observations may be taken as an indication of a still ongoing inflammatory reaction in this patient.

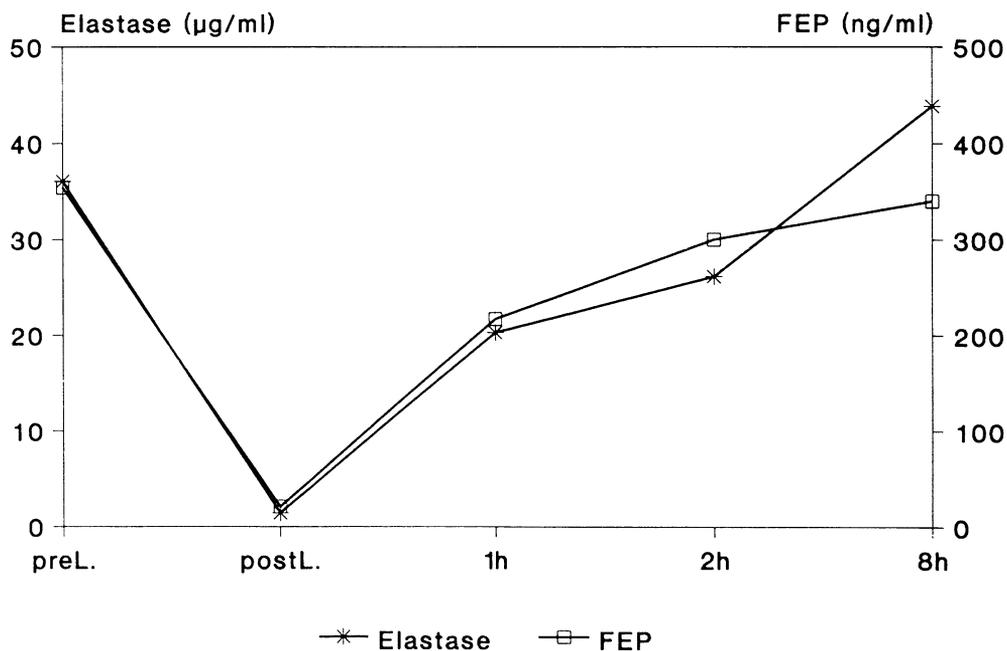


FIGURE 9. PMN elastase (in complex with α_1 -proteinase inhibitor) and fibrino-clastase-peptide (FEP) in exudate samples taken before (preL.) and immediately after (postL.) lavage of the abdomen of a peritonitis patient with Ringer lactate solution and in drainage fluids collected between 0 to 1 h, 1 to 2 h, and 2 to 8 h after lavage.

3. Conclusion

Summarizing the data obtained in our clinical studies and those of other authors on patients suffering from multiple trauma and/or sepsis, it was clearly shown that the release of the phagocyte proteinases, PMN elastase and cathepsin B, beyond normal values evoked by the physiological turnover of the phagocytes as well as the activation of proteolytic clotting enzymes, closely correlated to onset and severity of the acute inflammatory reaction. Although not outlined in detail herein, the above-mentioned studies, investigating a causal relationship between the occurrence of these parameters in body fluids and the disease state, in most cases fulfilled all important decision tree questions formerly established by Neugebauer et al.⁵⁰ to verify Koch-Dale's criteria 1 and 2 for a substance being an inflammatory mediator or even effector.

The inflammatory effector role proved especially true for PMN elastase, because the destructive potency of this proteinase could be convincingly demonstrated at least on vital humoral proteins in relation to the propagation of organ dysfunctions in traumatized as well as septic patients. Moreover, the studies corroborated, without doubt, that in patients who have incurred severe trauma or infection, the underlying cellular and humoral biochemical events are quite similar regardless of the etiology of the insult. Thus, the clinical outcome seems to be due to the magnitude of the inflammatory response, which of course is highest if trauma is superimposed by infection.

Furthermore, an excessive local (α_1 PI) and systemic (AT III) consumption of proteinase inhibitors occurs as a main consequence of the release of lysosomal phagocyte proteinases and the activation of proteolytic blood cascade enzymes during severe inflammatory reactions. Since this entity seems to be the most critical cause which allows the proteolysis-induced (multiple) organ damage, supplementation of the body's inhibitor potential by

exogenous proteinase inhibitors — isolated from human material or produced by gene technology as discussed by Fritz et al.⁵¹ — should be a highly promising therapeutic approach in traumatized and septic patients.

B. PHAGOCYTE PROTEINASES IN EXPERIMENTAL SEPTIC/ENDOTOXIC SHOCK

Although novel therapeutic approaches ultimately require validation in well-controlled clinical trials, it is virtually always necessary to obtain preliminary data in animals before applying new drugs or devices in humans. Thus, progress in sepsis research continues to depend upon studies using clinically relevant animal models and fulfilling commonly accepted criteria of the sepsis syndrome. Being applicable to both human and laboratory studies, the working definition of the sepsis syndrome comprises a constellation of clinical and laboratory findings indicative of a generalized inflammatory response leading to acute organ dysfunctions often, but not invariably, associated with the presence of serious bacterial, fungal, or viral infection. Furthermore, the septic shock phase is characterized by the circulatory decompensation (i.e., hypotension) due to an exacerbation of the septic syndrome.

To achieve such a clinic-like situation in an experimental setting, a variety of different approaches have been performed (extensively reviewed by Fink and Heard⁵²). Out of those, intravenous infusions of viable bacteria or of endotoxins (the main toxic cell wall constituents of Gram-negative bacteria) belong to the most common procedures. Although there are considerable doubts about the absolute clinical relevance of these approaches (e.g., because of differences in the hemodynamic responses depending on the amount of the deleterious agent and the application period) it turned out that experimental bacteremia or endotoxemia may provide a reasonable paradigm for sepsis in humans given the proviso that the animal model chosen adequately replicated those features of the clinical syndrome which should be explored in the experiment (e.g., release of phagocyte proteinases in relation to organ dysfunctions).

With respect to the relevant animal species selected in the different laboratory models of sepsis and septic shock, dogs, pigs, and nonhuman primates proved to be invaluable in the exploration of the underlying pathobiochemical mechanisms, because of the obvious similarities of these animals to humans concerning physiology and biochemical reactions.

1. Release of PMN Elastase and Cathepsin B

The release of granulocyte elastase and its interaction with plasma proteins was shown for the first time by Aasen and Ohlsson⁵³ in a controlled prospective study on dogs ($n = 6$) receiving a slow infusion of a lethal dose of *E. coli* endotoxin. During the endotoxin infusion, a marked decline in blood pressure and leukocyte counts was paralleled by a rapid increase of granulocyte elastase in complex with α_1 PI in circulating blood. Maximal values (determined by radial immunodiffusion) were reached at the end of the endotoxin infusion followed by continuously high levels up to the lethal outcome. Control animals ($n = 4$) infused only with sterile isotonic saline did not reveal such an increased release of PMN elastase. Antigen concentrations of the complement factor C3 in endotoxic dogs were gradually reduced to about 40% of the initial values at the end of the observation period. Moreover, crossed immunoelectrophoresis with antiserum against C3 indicated proteolysis of this complement factor and formation of related cleavage products. As reported elsewhere, the authors have also noted consumption of coagulation factors, reduced levels of components of the plasma kallikrein-kinin system and activation of the fibrinolytic system during the endotoxin experiments (review of literature in Reference 53). In control animals, only insignificant changes of these plasma proteins occurred. In a recent paper,⁵⁴ this research group published a pronounced structural identity of dog and human granulocyte elastase and the development of a specific ELISA test system for the determination of the canine elastase- α_1 PI complex

similar to that which we and others have used for human elastase. Confirming earlier results, slow intravenous infusion of a lethal dose of *E. coli* endotoxin in seven dogs over a period of 2 h evoked a drop in blood pressure simultaneously with a tremendous increase (20- to 30-fold that of normal) of complexed elastase in plasma between the 3rd and 12th h after starting the experiments. Plasma levels were much higher in the dogs that died during the observation period as compared to the survivors. Interestingly, concentrations of complexed elastase in plasma of normal control and endotoxic dogs, respectively, were strikingly similar to those of healthy humans or septic patients.

In contrast to dog and human granulocytes, porcine PMN leukocytes contain proteolytic as well as inhibitory activities. As shown by Geiger and colleagues (review of literature in Geiger et al.⁵⁵), the main inhibitory activity against lysosomal neutral serine proteinases such as elastase resides as a cytosolic serpin-like inhibitor, called leukocyte neutral proteinase inhibitor (LNPI). This inhibitor is released simultaneously with elastase due to stimulation or disintegration of the PMN cells and inhibits effectively this enzyme outside of the granulocytes. Thus, applying a specifically developed ELISA test system, quantification of the elastase-LNPI complex in porcine plasma turned out to be a reliable measure of the extracellularly discharged PMN constituents.⁵⁵ Mean plasma levels of this complex in healthy pigs reached 16 ± 11 ng/ml. In a preliminary study on eight septic pigs, levels up to 25 times higher were observed during the development of septicemia induced by 2-h infusion of live *E. coli*.⁵⁵ Maximal values were measured about 10 h after the end of the bacterial application. These results could be clearly confirmed in a second randomized trial, in which the release of the porcine PMN constituents was highly correlated with the occurrence of hypotension and hypoproteinemia due to infusion of viable bacteria.⁵⁶ In another prospectively controlled randomized, short-term study performed by our research group⁵⁷ a similar significant increase of PMN elastase in complex with LNPI above normal was noticed concomitantly to the development of organ dysfunctions in 18 pigs receiving a continuous infusion of *Salmonella abortus equi* endotoxin over the whole observation period of 6 h. No such changes occurred in sham-treated control animals.

In addition to the proof of PMN elastase release in septic/endotoxic situations, pigs are the only animal species in which an extracellular discharge of the monocyte/macrophage-derived cysteine proteinase cathepsin B above normal has been reliably demonstrated after endotoxin infusion so far.^{9,58} Starting from a normal level of about 200 mU/l (corresponding to fourfold that of normal values in healthy humans) intravenous infusion of *S. abortus equi* endotoxin for 6 h in 19 pigs evoked a steady increase of cathepsin B in plasma up to 3.5-fold that of normal at the end of the 6-h observation period.⁹ Concomitant to this increase, a significant decrease in arterial blood pressure occurred, indicating a severe septic shock state. In contrast, sham-treated control animals ($n = 7$) did not show any remarkable, pathophysiological changes. Similar results could be demonstrated in another randomized trial with 11 pigs receiving also a 6 hour-continuous infusion of *S. abortus equi* endotoxin.⁵⁸ In this study, the endotoxin-induced rise of cathepsin B was partially reduced in 13 animals treated with the platelet activating factor (PAF) receptor antagonist WEB 2086 compared with the 11 septic untreated controls indicating that the cathepsin B release may have been mediated to some extent by PAF in this experimental setting.

Due to obvious similarities between humans and baboons especially with regard to the antigenic identity of many plasma and cellular proteins, a modified version of the ELISA test system for human PMN elastase can easily be applied for quantification of this granulocyte proteinase in plasma of baboons. As recently shown by Redl et al.,⁵⁹ using this test system, continuous intravenous administration of live *E. coli* over 8 h in seven baboons evoked a massive release of PMN elastase from within 1 h after starting the experiment to the end of the observation period. Although normal plasma levels of extracellularly discharged PMN elastase in baboons reached only one third that of humans, the 20- to 30-fold increase in septic animals was highly comparable to the situation in septic patients.

As already reported by Egbring and Havemann (1978),⁶⁰ a bolus infusion of 50 g *S. abortus equi* endotoxin per kilogram of body weight in a green monkey induced not only the release of PMN elastase within 20 to 300 min after the endotoxin injection — as demonstrated by one-dimensional immunoelectrophoresis — but also a concomitant decrease in activity of several coagulation factors quite similar to findings in septic patients.^{37,38,60}

2. Eliciting Inflammatory Reactions by Exogenous Administration of Elastase

Because of ethical objections it is nearly impossible, under clinical conditions to verify the Koch-Dale criterion 3, which is eliciting pathophysiological reactions comparable to those seen during septic inflammation by the exogenous administration of a putative inflammatory mediator. Even in animal experiments such studies can be performed only with great limitations (e.g., insufficient amount of the isolated substance available). Thus, until now, just a few data were published concerning the assumed pathogenetic role of lysosomal proteinases exogenously applied to experimental animals.

In a very preliminary, early approach Egbring and Havemann⁶⁰ infused highly purified, endotoxin-free human PMN elastase (7 mg/kg body weight) in green monkeys. Immediately after the intravenous injection, elevated complexed elastase in plasma could be detected using a specific antihuman elastase antibody. Thereafter, the level steadily decreased until only minute amounts of the granulocyte enzyme could be seen at 6 h postinjection. After 24 h, the animals' circulation had been virtually cleared of the complexed human PMN elastase. Simultaneously, to the maximal increase of elastase, a pronounced inactivation of the clotting factors II, V, VIII, and (to a less degree) XII occurred. Reduction of fibrinogen and generation of its split products were somewhat delayed (maximum between 30 to 60 min), whereas factor XIII activity decreased over a period of 24 h. During the test period no changes in peripheral blood cell counts were observed, yet a moderate bleeding tendency appeared as was indicated by skin hematoma and bleeding gums. Although no further signs of a sepsis-like syndrome became obvious, presumably due to a too low and short-lasting exogenous elastase administration in these animals, the given changes can be taken as a first hint to the involvement of PMN elastase in sepsis-associated clotting disorders.

In contrast to the relatively limited data on proteinase-induced tissue damage of the lung in septic inflammation, intensive studies on pulmonary emphysema were performed several years ago by Janoff and colleagues^{61,62} as well as by many other researchers.⁶³ The results indicate that the "proteinase pathogenesis" of this chronic disease may be fairly comparable to that of the ARDS in septic MOF. Interestingly, in one of the studies,⁶¹ instillation of a certain amount of purified human PMN elastase in isolated-perfused dog lungs produced an anatomic emphysema nearly identical to that evoked by porcine pancreatic elastase. Non-elastolytic proteinases, including trypsin, chymotrypsin, and collagenase, failed to induce emphysematous changes in this test system. Moreover, localized endotracheal instillation of human PMN elastase or porcine pancreatic elastase, respectively, in living dogs revealed similar dose-dependent, proteolysis-elicited lesions of the lung tissue even in the presence of normal concentrations of circulating and local proteinase inhibitors. These data demonstrate a high functional identity of human PMN elastase with porcine pancreatic elastase despite an obvious divergent origin of the proteinases.

Taking advantage of this identical pathogenic potency of the two elastolytic lysosomal proteinases, Stokke and colleagues studied the effect of a 3 to 5 h continuous intravenous infusion of porcine pancreatic elastase (ca. 6.6 mg/kg bw/h) into normal (n = 9) or agranulocytic (n = 4) minipigs concerning the development of ARDS and blood coagulation disorders.⁶⁴⁻⁶⁶ Since in normal and agranulocytic minipigs the continuous intravenous infusion of elastase led to an early equal impairment of pulmonary hemodynamics and gas exchange functions, the authors assumed that these effects may be mediated by the elastase-induced activation of other cascade systems (e.g., complement factors and arachidonic acid metab-

olites) rather than by elastase itself. Moreover, increase in capillary permeability, evoking an ARDS-like interstitial edema, was prevented in agranulocytic pigs, whereas in normal animals elastase infusion induced a massive pulmonary granulocytosis combined with the development of interstitial edema and progressive respiratory failure. Thus, the release of further proteinases and other inflammatory mediators such as toxic oxygen compounds from sequestered granulocytes either directly or indirectly stimulated by the exogenous elastase — may have led to the destruction of the lung tissue.

In addition, pancreatic elastase infusion in normal minipigs caused a pronounced hypocoagulability with degradation of the clotting factors V, VII, VIII, X, XIII, fibrinogen, and AT III, whereas in granulocyte-depleted animals mainly factor XIII and AT III were consumed at the end of the short-term experiments. These results can be taken as a further hint to the proteolytic potency of activated granulocytes under pathological conditions.

Since all measured parameters remained unchanged in a control group ($n = 3$), the given data clearly indicate that elastase (mainly of PMN origin) may be involved in the pathophysiology of acute lung failure and defects in the blood coagulation seen during the septic-like syndrome in animals and humans. But obviously, the PMN elastase is only one inflammatory factor in a very complex mechanism of interactive mediator-releasing cascade systems.

3. Conclusion

As demonstrated in the preceding sections, the demands for being a causal chemical factor in the sepsis syndrome according to the Koch-Dale criteria 1 and 2, which mean presence of an inflammatory mediator in disease and its absence (beyond normal physiological concentrations) in health,⁵⁰ could be confirmed for PMN elastase and monocyte/macrophage-derived cathepsin B not only in humans but also in several animal models on experimental septic/endotoxin shock. Moreover, administration of animal exogenous elastases (of PMN cell or pancreatic origin) elicited pathophysiological reactions similar to those observed during septic inflammation. Thus, not disregarding the limitations of the underlying experiments, at least PMN elastase fulfilled the Koch-Dale criterion 3 for being a putative, although not the sole, causal inflammatory mediator in such a disease state.

C. PROTEINASE INHIBITION AS A SUITABLE THERAPEUTIC APPROACH IN ACUTE INFLAMMATION

To further establish the ultimate relevance of a putative causal mediator in acute inflammation such as septic shock, the use of specific mediator antagonists as therapeutic tools should prevent or at least reduce to some extent severe signs of inflammation in accordance with Koch-Dale criterion 4 as indicated by Neugebauer and Lorenz.⁶⁷ Since especially PMN elastase turned out to play a major role in shock-associated organ and hemostasis dysfunctions, accomplishing the demands of the first three Koch-Dale criteria, we and others envisaged the therapeutic application of elastase inhibitors as the most promising approach to interfere with proteolysis-induced pathomechanisms in acute inflammation.^{8,51,68} Moreover, the excessive consumption of AT III during severe inflammatory reactions, which enables an unrestricted activity of hemostatic proteinases further intensifying the overall pathological proteolysis, should be compensated by the exogenous administration of potent thrombin inhibitors.^{69,70}

Yet, before applying such drugs in humans, especially those of nonhuman origin generated by gene technology or chemical synthesis,⁵¹ safety and efficacy first has to be demonstrated in convenient animal models.

1. Inhibitors of PMN Elastase or Thrombin in Experimental Septic/Endotoxic Shock

In a preliminary, controlled study on sepsis in young pigs, the prophylactic application of the relatively specific recombinant elastase inhibitor, eglin c (originally isolated from the

leech *Hirudo medicinalis*), caused a significant reduction in the consumption of AT III and other plasma proteins as well as in the formation of interstitial pulmonary edema (Jochum et al.⁷¹). As assessed by measurement of arterial blood pressure and total protein concentration in plasma, in a more extended study, Siebeck et al.^{56,72} demonstrated that eglin c can also reduce hypotension and the overall capillary leakage induced by the infusion of live *E. coli* in pigs. In this study, significantly lower concentrations of the elastase-LNPI complex in plasma were measured under eglin c administration, presumably indicated an extremely rapid competition of the exogenous elastase inhibitor with the granulocyte-derived serine proteinase antagonist LNPI.⁵⁶ Such a rapid complexation by eglin c seems to prevent proteolytic activity of released elastase more effectively than by the endogenous inhibitor.

Moreover, in another controlled investigation, Siebeck, Hoffmann, and colleagues,^{57,73} showed that besides eglin c, the thrombin-specific inhibitor hirudin — another recombinant inhibitor also formerly isolated from the medical leech — significantly improved endotoxin shock syndromes in minipigs. Fibrinogen consumption, formation of fibrin monomers as well as the occurrence of pulmonary vasoconstriction and the release of PMN constituents (elastase, LNPI) were clearly lower in endotoxemic animals treated prophylactically with hirudin as compared to those without continuous intravenous inhibitor infusion. Since hirudin does not interfere with PMN elastase inhibition by endogenous inhibitors, the conclusion can be drawn that the activation of clotting enzymes, which are known as potent, direct or indirect stimulators of the PMN cells,⁷⁴⁻⁷⁶ was effectively reduced by inhibition of thrombin, thus also lowering the stimulation of PMN granulocytes.

Interestingly in another randomized porcine endotoxin shock model, the supplementation of a purified AT III-heparin complex had only a slight, nonsignificant positive effect on the endotoxin-induced mortality and the oxygen saturation in arterial blood (as an indication of pulmonary function), even though the consumption of fibrinogen and the formation of soluble fibrin monomers were clearly prevented in the drug-treated animals (Spannagl et al.⁷⁷). Furthermore, prothrombin consumption was similar in the treatment and placebo groups suggesting that local thrombin generation, via factor Xa and binding of both enzymes to endothelial cell membrane receptors, may protect them from inhibition by the AT III-heparin complex. On the other hand, the applied amount of the AT III-heparin complex may not have been sufficient to inhibit the activation of contact phase enzymes (plasma kallikrein, factor XIIa) which are supposed to be potent stimulators of PMN granulocytes^{74,75} as previously mentioned. Therefore, the inflammatory process might have been maintained primarily via the release of phagocyte proteinases and reactive oxygen metabolites. Moreover, since after an initial rise (up to about 130%), the AT III activity decreased during further drug application while the antigen level still increased, the heparin in the complex may have also facilitated the inactivation of AT III by released PMN elastase (Jordan et al.³⁵) thereby lowering the inhibitory capacity under an otherwise presumably effective threshold level.

The requirement of high levels of AT III inhibitory activity in the circulation is also confirmed by results of Emerson et al.⁷⁸ concerning the efficacy of AT III supplementation in several animal models (rat, sheep, and baboon) of fulminant *E. coli* endotoxemia or bacteremia. Only very high dosage (up to threefold of normal) and prophylactic administration of AT III prevented organ damage and increased permanent survival in the experimental animals. Interestingly, the combined application of AT III and α_2 PI showed a significant, synergistic improvement of the pulmonary function compared to the single drug treatment in the endotoxemic sheep model. This indicates again that a complex interaction of lysosomal and humoral blood cascade proteinases contributes to the perpetuation of a septic-like inflammation.

2. Antithrombin III Supplementation in Clinical Sepsis

Beside the therapeutic animal experiments, preliminary clinical studies (reviewed in References 69, 70, and 80) have also proven the postulated positive effects of thrombin

inhibition on the hemostatic system by AT III supplementation in severely ill patients. Yet, a statistically significant reduction in MOF or lethality compared to untreated patients could be verified in only a few trials. Since in these studies just normal plasma levels of AT III were achieved by the applied therapeutic regimen infusing purified human AT III concentrates, we draw the conclusion that only AT III levels well above the normal plasma range may be able to interfere with phagocyte-derived inflammatory reactions, thus improving not only disseminated intravascular coagulation but also organ dysfunctions in clinical sepsis. In addition, this assumption is especially supported by our animal experiments⁷⁷ and those of Emerson et al.⁷⁸

Therefore, we conducted a prospective, randomized study (clinically directed by Dr. Inthorn, Surgical Clinic Großhadern) on septic patients with the aim to increase the AT III inhibitory activity at least above 120% of normal. To achieve this, AT III concentrates were intravenously infused twice daily over 21 d according to a modified regimen originally described by Blauhut et al.⁸¹ Blood samples were taken twice daily throughout the entire observation period. Preliminary data and a detailed outline of the performance of the still ongoing trial have been published by Jochum et al.⁸² Here, only the most important results will be presented. Up to now, 15 patients — fulfilling previously mentioned septic criteria — could be enrolled in each of the groups (control and therapy).

With our application scheme, the AT III activity in the treatment group was elevated to mean plasma levels slightly below 120% during the first 9 d, followed by an increase above 120% thereafter, whereas in the control group levels between 60 (early phase) and 80% (later phase) were measurable. Although all AT III-treated patients received nearly the same amount of the inhibitor concentrate (between 8000 and 4000 U/d), those individuals who survived the septic event showed clearly higher AT III levels (up to a mean of 135% during the first 5 d after sepsis diagnosis) than patients who died despite of AT III supplementation (mean AT III activity between 100 and 115% in the early septic phase). Thus, immediate AT III substitution in sufficiently high amounts after early diagnosis of a septic episode appears to be of great importance in improving the survival of the patients.

Probably due to a too-late onset of the inhibition therapy in addition to the application of still insufficiently high AT III dosages in some of our patients, the overall mortality could be only reduced from 87% in the control group to 60% in the AT III-treated collective. This diminution in lethality was statistically nonsignificant, yet a clear improvement of organ functions — especially of lung, liver, and kidney — in the treated patients as well as a further deterioration in the control group became evident.

Mean plasma levels of complexed elastase were elevated up to sixfold of normal upon admission and decreased gradually to about threefold in both groups until the end of the observation period. Although there were no statistically significant differences, a slight trend to lower plasma elastase levels appeared in the AT III group. Similar minute distinctions were seen in AT III-treated patients who survived compared to those who died. An obvious reduction of complexed elastase in plasma due to AT III supplementation was demonstrated by Seitz et al.⁷⁷

In our study, plasma prekallikrein and prothrombin levels were reduced up to 30 and 50%, respectively, upon sepsis diagnosis and showed a more or less pronounced change to higher values (40 and 80% of normal) later without distinct differences in the placebo and treatment groups. Interestingly, however, prekallikrein levels rose steadily to 50% in AT III-treated survivors, whereas in the nonsurvivors a further transient decrease to 20% occurred. An analogous behavior was observed for prothrombin which reached 80% of normal 10 d after onset of sepsis in the AT III-treated survivors, while in the moribund patients plasma prothrombin levels as low as 60% were measured at that time. Thus, the rise in AT III inhibitory activity to nearly 140% in the early septic phase may have been beneficial enough to preserve clotting proenzymes from excessive activation throughout a septic period. Nevertheless, it became obvious from our findings that a convincing improvement of the

overall septic state deserves an even higher and earlier substitution of AT III than that applied in this study.

3. Conclusion

The given data derived from the therapeutic experimental and clinical studies on acute inflammation unequivocally indicate that proteolytic pathomechanisms due to phagocyte and hemostasis proteinases play an important role in the onset and perpetuation of inflammatory processes such as sepsis-induced multiple organ dysfunctions. Therefore, plasma levels of regulatory elastase and thrombin inhibitors have to be elevated by a suitable supplementation and kept well above the normal inhibitory activity in healthy people to achieve a significant improvement of the clinical situation in severely ill patients.

Yet, despite the fact that the leech-derived elastase and thrombin inhibitors, eglin c and hirudin, have shown a high efficacy in animal experiments, their clinical application has to be considered with caution, especially concerning a putative antigenicity of nonhuman proteins in long-term studies. Thus, a therapeutic approach with isolated, purified human plasma inhibitor preparations (α_1 PI, AT III) is the only clinical choice at present.

Although the natural sources for the isolation of proteinase inhibitors from human material are very limited, the design of highly effective inhibitory proteins on the basis of human inhibitor molecules by molecular modeling and their production by recombinant DNA technology is the most promising approach to get the quantities necessary for proteinase inhibition therapy in the future (Fritz et al.⁵¹).

IV. SUMMARY

As outlined in detail by Neugebauer et al.,⁵⁰ several years ago, more than 100 different mediators were described as being causally related with acute inflammation and circulatory shock. Despite, or probably because of, the fact that for none of these factors unequivocal proof of its ultimate relevance had been given until now, there is still a growing number of new mediators which are supposed to play the central role in an acute inflammatory process such as multiple trauma and sepsis. Yet, to establish a cause-effect relationship of a single mediator in shock the classical criteria of Koch-Dale (being a causal chemical factor in a pathological reaction) should be considered in a modified version using the method of meta-analysis and models of decision trees as suggested by Neugebauer et al.⁵⁰ and Neugebauer and Lorenz.⁶⁷

Being aware of the fact that not a single factor, but a mixture of causal inflammatory mediators is acting in concert to evoke a sepsis-like syndrome following severe trauma, major surgery, or infection, we have been especially interested in the extent proteolytic pathomechanisms may participate in the initiation and perpetuation of these acute inflammatory processes. Although we did not use meta-analysis procedure and models of decision trees in detail, a more or less narrative review of our research work and that of other authors with respect to the topic of interest revealed a prominent role of proteinases in acute inflammation. This statement is based on the following indications:

1. The release of the lysosomal phagocyte proteinases, PMN elastase, and monocyte/macrophage-derived cathepsin B, as well as the activation of proteolytic blood cascade enzymes in correlation to the severity of trauma-, surgery-, and/or bacteria-induced inflammation could be clearly verified in a variety of clinical and experimental animal studies. Moreover, in humans, the amounts of the extracellularly discharged phagocyte proteinases in plasma at an early stage of the disease were highly predictive for forthcoming organ failure and ultimate outcome.

2. The consumption of proteinase inhibitors (α_1 PI, AT III) and other plasma proteins susceptible to proteolytic degradation coincided with the occurrence of proteolytic activity, especially that of PMN elastase.
3. Split products of functional proteins specifically generated by the phagocyte proteinases could be demonstrated in local and systemic human body fluids in correlation to the extracellular release of these enzymes.
4. Exogenous administration of PMN elastase and porcine pancreatic elastase in animals evoked pathophysiological reactions similar to those observed during septic inflammation.
5. The therapeutic use of specific PMN elastase or thrombin inhibitors in experimental septic/endotoxin shock or clinical sepsis, respectively, prevented or at least reduced to some extent severe signs of inflammation.

Yet, despite these convincing data the ultimate assessment of the relevance of proteolytic enzymes in shock states notwithstanding the numerous other candidates, requires the appliance of the probabilistic model suggested by Neugebauer and Lorenz⁶⁷ exhibiting the factor of interest as a necessary, sufficient, or contributory determinant.

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