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# PROTEINASE ACTION

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CONTENTS

PROTEINASE INHIBITORS

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# PROTEINASE-PROTEINASE INHIBITOR IMBALANCE IN INFLAMMATION AND MULTIPLE TRAUMA WITH SPECIAL EMPHASIS ON RELEASE OF GRANULOCYTIC LYSOSOMAL ELASTASE*

JOCHUM M.[1], DUSWALD K.-H.[2], DITTMER H.[2] and FRITZ H.[1]

[1]Department of Clinical Chemistry and Clinical Biochemistry* in the
[2]Surgical Clinics of the University of Munich
Munich, FRG

## INTRODUCTION

Severe injury or infection trigger the so-called inflammatory response of the organism. This response includes besides other reactions the activation of humoral systems such as clotting, fibrinolysis, complement and kallikrein-kinin cascades, and of various cellular systems, especially of phagocytes, mast cells and lymphocytes, but also of stress hormone producing cells.

Phagocytes such as polymorphonuclear granulocytes and macrophages contain high numbers of lysosomes equipped with a powerful hydrolytic or proteolytic potential (Dingle, 1977). Normally the cell uses this enzyme equipment, in addition to oxidizing agents, for mainly two purposes (Klebanoff and Clark, 1978):
(i) the maintenance of the intracellular protein catabolism including the degradation of wasted endogenous substances, and
(ii) the defense of invasive organisms by degradation after phagocytosis of viruses and bacteria.

If released extracellularly, they may enhance the inflammatory response via two major routes (Fig. 1):

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* An extented version of this article will be published in Clinical Chemistry (H. Fritz et al.: Granulocyte Proteinases as Mediators of Unspecific Proteolysis in Inflammation. A Review).

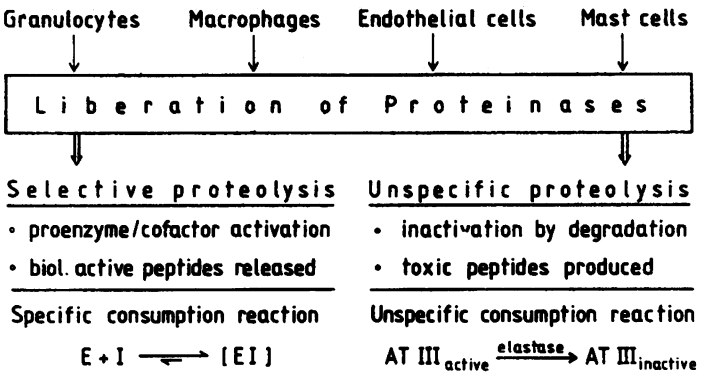


Fig. 1. Proteolytic processes due to liberation of lysosomal proteinases from various body cells.

Selective proteolysis leads to proenzyme and/or cofactor activation including the formation of biologically highly potent peptides, as for example kinins and anaphylatoxins. The proteinases formed are eliminated by specific interactions with their target inhibitor proteins. Hence, reaction mechanisms of high selectivity are responsible for the so-called specific consumption of factors of the clotting, fibrinolysis, complement and kallikrein-kinin cascades.

Unspecific proteolysis means inactivation of soluble factors or destruction of structural elements simply by proteolytic digestion. Such unspecific consumption reactions may be accompanied also by the production of toxic peptides like, for example, the clot formation inhibiting fibrin/fibrinogen degradation products.

## LYSOSOMAL PROTEINASES

Of the lysosomal proteinases known so far, elastase and

cathepsin G, the neutral proteinases from polymorphonuclear granulocytes, deserve special interest. Like the acidic thiol and aspartate proteinases, the various cathepsins, they are stored in the lysosomes in fully active form. The most striking feature of both, elastase and the chymotrypsin-like cathepsin G, is an almost unlimited cleavage specifity (Fig. 2).

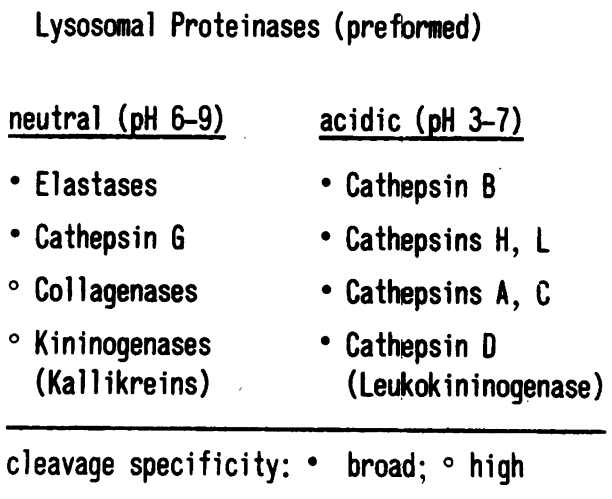


Fig. 2. Lysosomal proteinases of neutrophils.

They are able to degrade numerous humoral factors including proteinase inhibitors as well as structural elements like elastin and collagen type III and IV at physiological pH. The potential lysosomal digestion capacity in humans is clearly demonstrated by the calculated synthesis rate of more than 1 g of neutral proteinases per day. However, inside the cells, the lysosomal proteinases are kept under control by either assemblage in membrane coated organelles or by proteinase inhibitors present in the cytosol.

## PLASMA PROTEINASE INHIBITORS

Lysosomal proteinases escaping from their natural target cells are faced usually with potent antagonists, the wellknown proteinase inhibitor proteins (Travis and Salvesen, 1983).

$\alpha_2$-Macroglobulin ($\alpha_2$M) inhibits effectively serine proteinases as well as thiol, aspartate and metallo proteinases; due to its high molecular weight, its function is normally restricted to the vascular bed.

$\alpha_1$-Proteinase inhibitor ($\alpha_1$PI), the major antagonist of the lysosomal neutrophil elastase, is present in remarkable high concentration in blood, but occurs also in interstitial fluid and mucous secretions.

$\alpha_1$-Antichymotrypsin ($\alpha_1$AC), a rapidly responding acute phase reactant - it can reach up to six times its normal concentration during the inflammatory response - is a potent inhibitor of lysosomal neutrophil cathepsin G and mast cell chymase.

Compared to $\alpha_1$PI and $\alpha_1$AC, the plasma concentrations of the other given inhibitors are clearly lower. Nevertheless, the proteinase inhibitor proteins represent approximately 60 % of the residual plasma proteins after removal of albumin and the immunoglobulins. This is an indirect indication upon the significance of proteinase inhibitors as regulatory proteins of the organism.

The association between known plasma proteinase inhibitors and their target enzymes is schematically compiled in Fig. 3.

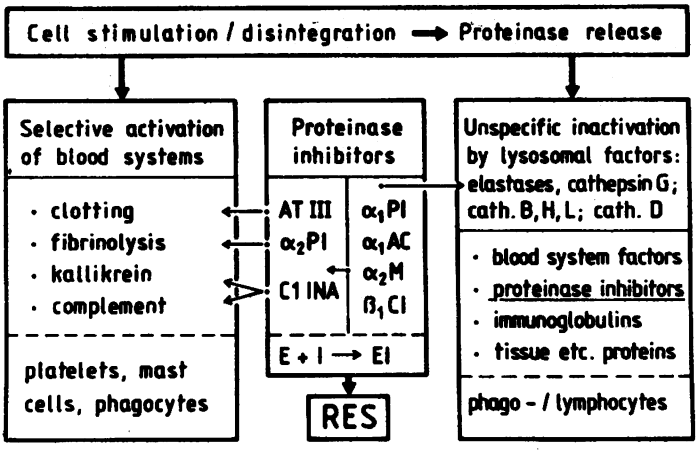


Fig. 3. Activation and consumption reactions: specific activation of blood systems by system-specific proteinases (left part); unspecific degradation of plasma factors by lysosomal proteinases (right part); complex formation with proteinase inhibitors and elimination of the enzyme-inhibitor complexes by phagocytes of the reticulo endothelial system (RES; central part).

Excessive activation of blood systems may be controlled primarily by three inhibitors: antithrombin III (AT III) regulates clotting, $\alpha_2$-plasmin inhibitor ($\alpha_2$PI) fibrinolysis, and C1-inactivator (C1-INA) both, the classical complement pathway and the intrinsic coagulation cascade, the latter by inhibition of plasma kallikrein and Hageman factor, respectively its low molecular weight fragment.

The occurrence of complexes between $\alpha_2$-macroglobulin and plasma kallikrein or plasmin in plasma under certain pathological conditions indicates that this multifunctional glycoprotein is also involved in the regulation of the blood system cascades. However, present evidence suggests a predominant protective

role of $\alpha_2$-macroglobulin in prevention of unspecific proteolysis by inhibition of all types of liberated lysosomal proteinases. Remarkable, in humans $\alpha_2$M is, in contrast to $\alpha_1$PI and $\alpha_1$AC, not an acute phase reactant, although it is supposed to be the most important endogenous plasma proteinase inhibitor.

Inhibition of activated or liberated proteinases by the given inhibitors means that formation of vasoactive or toxic peptides such as kinins or anaphylatoxins as well as proteolysis-induced stimulation of cellular systems like, for example, thrombin-induced platelet aggregation or the anaphylatoxin-induced chemotactic response of granulocytes, is also depressed. Therefore, reduction of the proteinase inhibitory potential of the organism by unspecific proteolysis represents one of the most striking pathological effects caused by lysosomal proteinases. As we could show some years ago, AT III, for example, is rapidly inactivated in the presence of catalytic amounts of neutrophil elastase (Jochum et al., 1981 a).

The same holds true for $\alpha_2$PI and C1-INA (Brower and Harpel, 1982). Even $\alpha_1$PI, the major antagonist of neutrophil elastase, can be proteolytically inactivated by a lysosomal metallo enzyme from macrophages (Banda et al., 1980). Moreover, oxidation of the methionine residue in the enzyme-reactive site of $\alpha_1$-proteinase inhibitor leads to a significant reduction of the affinity of this inhibitor to neutrophil elastase (Beatty et al., 1980). Such oxidizing agents, for example, superoxide, hydroxyl radicals and hydrogen peroxide, are produced in high amounts in the phagolysosomes to facilitate intracellular protein breakdown and may be released simultaneously with the lysosomal enzymes under pathological conditions (Klebanoff and Clark, 1978).

Hence, severe injury or infection can induce consumption of proteinase inhibitor proteins via three pathways:

(i) complex formation with liberated lysosomal or activated plasma proteinases,
(ii) inactivation by proteolytic degradation, and
(iii) inactivation by oxidative denaturation.

The latter mechanism deserves special interest in view of the proposed pathological effectiveness of neutrophil elastase. Although the oxidized $\alpha_1$-proteinase inhibitor is still capable to react slowly with neutrophil elastase, the complex thus formed is readily dissociated by substrates exhibiting high affinity for elastase, for example, elastin, that means, the oxidized inhibitor cannot hinder elastase to digest natural target substrates. Remarkable, in contrast to the native inhibitor, oxidized $\alpha_1$-proteinase inhibitor does not react at all with pancreatic elastase.

## RELEASING PROCESSES AND MEASUREMENT OF LIBERATED LYSOSOMAL PROTEINASES

Most intriguing is the question concerning the mechanisms responsible for a more or less dramatic escape of lysosomal factors from within the cells (Fig. 4).

Whereas only small amounts should leak out during normal phagocytosis, relatively high amounts could be excreted during frustrated phagocytosis when the phagocyte is unable to take up a comparatively huge structural element, as for example a vascular plasma membrane or parts of cartilage. Disintegration processes caused by endogenous or exogenous endotoxins, eventually in combination with complement lysis, are most dramatic events leading to release of all lysosomal or phagolysosomal constituents.

Neutrophil elastase liberated under such conditions is present in the circulation primarily in form of the elastase-$\alpha_1$-proteinase inhibitor (E-$\alpha_1$PI) complex (Fig. 5). A small amount of neutrophil elastase may be bound also to $\alpha_2$-macroglobulin ($\alpha_2$M), however, compared to the E-$\alpha_1$PI complex the E-$\alpha_2$M complex is

much more rapidly eliminated from the circulation ($t_{1/2} \sim 10$ min versus $t_{1/2} \sim 1$ h) so that estimation of E-$\alpha_2$M complexes in plasma needs special assays of extreme sensitivity.

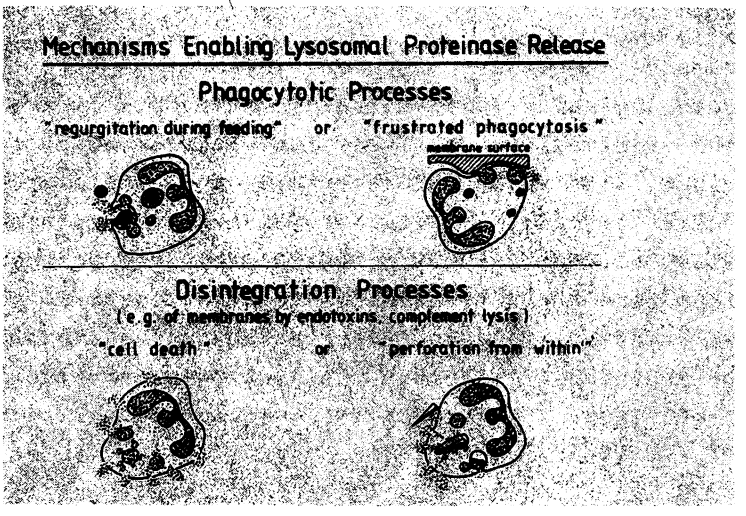


Fig. 4. Various releasing processes of lysosomal proteinases from within the cell.

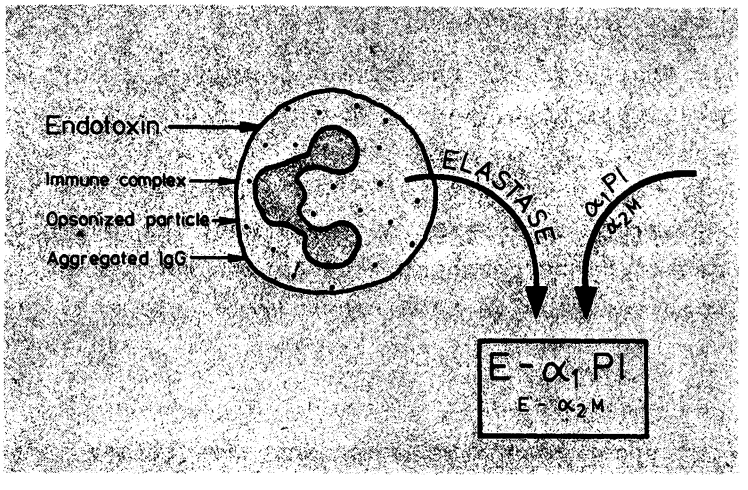


Fig. 5. Liberation of lysosomal elastase (E) due to different stimuli and complex formation with $\alpha_1$-proteinase inhibitor ($\alpha_1$PI) as well as (to a much less extent) $\alpha_2$-macroglobulin ($\alpha_2$M).

E-$\alpha_1$PI levels (Fig. 6) can be measured exactly with a newly developed enzyme-linked immunoassay (Neumann et al., 1981). Briefly, plasma samples or other body fluids are incubated in plastic tubes coated with sheep antibodies directed against human neutrophil elastase. After two washing steps, the surface-fixed E-$\alpha_1$PI complex is exposed to rabbit antibodies directed against human $\alpha_1$PI. These antibodies are labelled with alkaline phosphatase. Under the conditions used, the amount of bound alkaline phosphatase is proportional to the concentration of E-$\alpha_1$PI in the applied sample.

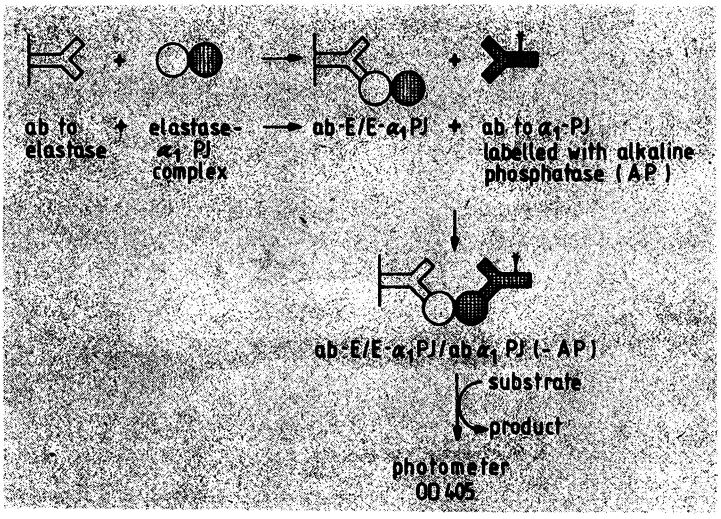


Fig. 6. Principle of the enzyme-linked immunoassay used for determination of the elastase-$\alpha_1$-proteinase inhibitor complex (E-$\alpha_1$PI).

ab-E = antibodies against human granulocytic elastase
ab-$\alpha_1$PI (-AP) = antibodies against human $\alpha_1$-proteinase inhibitor, labelled with alkaline phosphatase.

## CLINICAL STUDIES

Multiple organ failure during severe inflammation or after polytrauma concerns primarily lungs, liver and kidneys. These organs are especially rich in the formerly mentioned cells containing high numbers of lysosomes. In addition, rapid accumulation of polymorphonuclear granulocytes in the lungs during the inflammatory response is also a well-known phenomenon. Hence, a connection between the clinically observed sequence of organ failure and the inherent lysosomal functional capacity of an organ should be seriously considered.

### Neutrophil elastase release after major abdominal surgery

In our first prospective clinical study the plasma levels of the E-$\alpha_1$PI complex were measured in suitable intervals in more than 120 patients subjected to major abdominal surgery followed either by uncomplicated recovery or infection. Of the latter group, only 30 patients fulfilled previously defined and generally accepted septic criteria (Jochum et al., 1983). Of these patients fourteen survived the infection (group B), whereas sixteen died due to severe septicemia (group C). The eleven patients of the control group A recovered from the operation without complications.

Compared to healthy individuals or preoperative values (below 110 ng/ml), the operative trauma caused an up to threefold increase of the E-$\alpha_1$PI plasma levels (Fig. 7).

The elevated preoperative mean value of group C was caused by 6 patients being infected already before operation; the slight early postoperative decrease was probably due to surgical elimination of the infection focus.

In contrast to group A, group B and C patients maintained moderately elevated E-$\alpha_1$PI levels for several days after operation. At the time of diagnosis of septicemia, highly significantly elevated E-$\alpha_1$PI levels were measured corresponding to

an up to sixfold respectively tenfold increase in group B and C. Individual peak levels were found to be as high as 2 500 ng/ml in both groups.

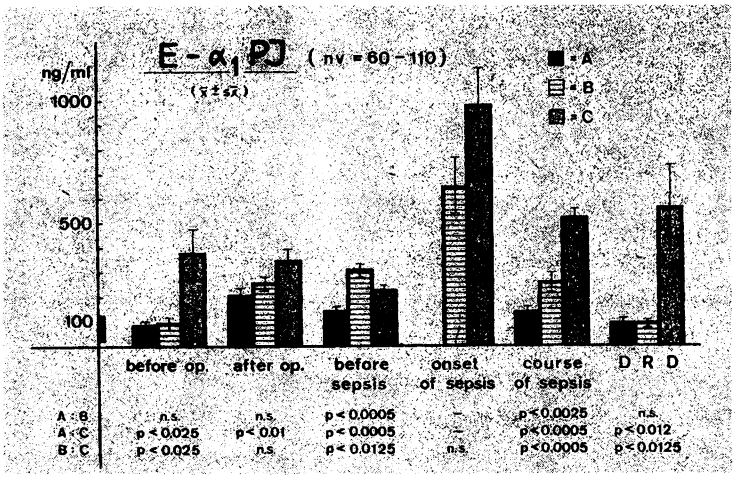


Fig. 7. Plasma levels of elastase-$\alpha_1$-proteinase inhibitor complex (E-$\alpha_1$PI) in patients subjected to major abdominal surgery.

A = patients (n = 11) being without postoperative infection
B = patients (n = 14) surviving postoperative septicemia
C = patients (n = 16) dying as a result of septicemia.

The E-$\alpha_1$PI levels are given as mean values ($\pm$ SEM) for the day before operation, the day after operation as well as for the postoperative phase before sepsis, at onset of sepsis and during septicemia. Last determination were done on day of discharge (D) for group A, on day of recovery (R) for group B, and before death (D or †) for group C.

nv = normal value

Most interestingly, in patients with persisting septicemia the E-$\alpha_1$PI levels remained high until lethal outcome (group C) whereas recovery from septicemia was reflected by a concomitant decrease of the E-$\alpha_1$PI levels to the normal range.

Parallel to the E-$\alpha_1$PI levels the plasma concentrations of other factors, expected to show a typical response to inflammatory stimuli, were also measured. The concentration pattern of antithrombin III, the most important regulatory inhibitor protein of the clotting cascade, turned out to be just opposite to that of the E-$\alpha_1$PI complex (Fig. 8). Especially at onset of sepsis and during septicemia, antithrombin III levels are reached, which have to be considered as clinically critical as far as the risk of hypercoagulability or disseminated intravascular coagulation is concerned.

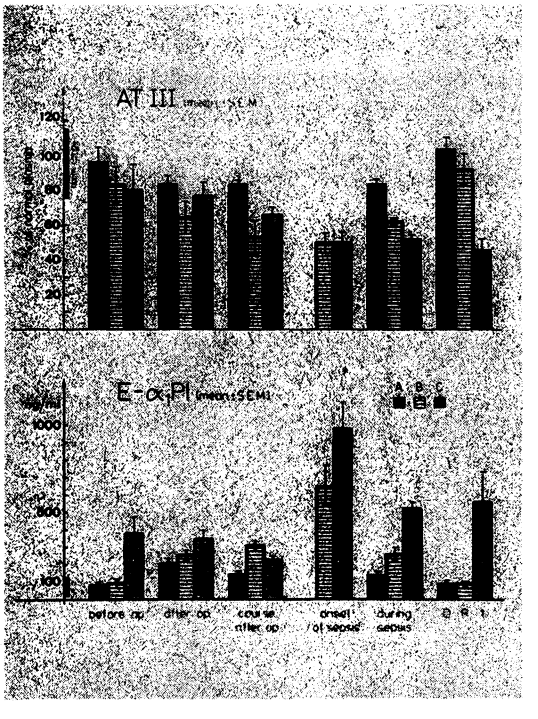


Fig. 8. Plasma levels of the inhibitory activity of antithrombin III (AT III) compared to the amount of the elastase-$\alpha_1$-proteinase inhibitor complex (E-$\alpha_1$PI) in patients subjected to major abdominal surgery.

For details, see legend to Fig. 7.

The concentration patterns found for coagulation factor XIII (F XIII), the fibrin stabilizing transglutaminase, and $\alpha_2$-macroglobulin were very similar to that of antithrombin III, as summarized in Fig. 9:

**Plasma levels, elevated (↑) or decreased (↓):**
**(↑↑) highly signif. (↑) signif. (n) normal**

| Parameter | | Sepsis | Prefinal | Survival |
|---|---|---|---|---|
| E-$\alpha_1$ PI complex | c | ↑↑ | ↑↑ | n |
| Antithrombin III | a | ↓↓ | ↓↓ | n |
| Factor XIII | a | ↓↓ | ↓↓ | n |
| $\alpha_2$-Macroglobulin | a | ↓↓ | ↓↓ | n |
| | c | ↓↓ | ↓↓ | n-↓ |
| C-reactive protein | c | ↑↑ | ↑↑ | n |
| $\alpha_1$-Proteinase inhibitor | a | n-↓ | n-↓ | n-↓ |
| $\alpha_2$-Plasmin inhibitor | a | n | n | n |
| $\alpha_1$-Antichymotrypsin | c | ↑ | n | n |
| C1 Inactivator | a | n-↓ | n | n-↓ |
| | c | n | n | n |

[a] activity assay [c] concentration assay

Fig. 9. Correlation between plasma levels of E-$\alpha_1$PI and other plasma factors in patients suffering from septicemia after major abdominal surgery.

High levels of E-$\alpha_1$PI were found at onset of sepsis and prefinal; these values normalized in survivors. Simultanously, low levels of AT III, $\alpha_2$M and F XIII occurred in sepsis and prefinal, whereas they normalized in survivors. The levels of the acute phase reactant C-reactive protein (CRP) paralleled mainly the E-$\alpha_1$PI, but turned out to reflect the course of sepsis much less specifically.

In contrast, the other given proteinase inhibitors did not show significant correlations to E-$\alpha_1$PI. This behaviour might be especially caused by the fact that these proteinase inhibitors

are acute phase reactants as well. Therefore, the consumption of the inhibitors due to complex formation with their target enzymes or other inactivation reactions might be compensated by the enhanced production of these proteins during inflammation.

Summing up, the results of this clinical study clearly showed that in inflammatory diseases a correlation exists between the release of a lysosomal enzyme marker, the neutrophil elastase, and the clinical situation of the patient, respectively the consumption of selected plasma factors. We take this as a clear indication that liberated lysosomal factors and especially neutrophil proteinases contribute significantly to the inflammatory response of the organism by unspecific degradation of plasma and other factors. This view is also supported by results of experimental animal studies performed more recently (Jochum et al., 1981b). Early application of potent exogenous proteinase inhibitors could prevent or at least diminish highly significantly endotoxin-induced consumption of various plasma factors including AT III and F XIII.

Neutrophil elastase release after polytrauma

In a preliminary study release of granulocytic elastase into plasma was followed up every 4 hours in patients who suffered from multiple trauma. The increase of the E-$\alpha_1$PI complex up to 16 hours after accident coincided clearly with the severity of the injury (Fig. 10), the degree of injury being established on the basis of a hospital internal scale ranging from 1 - 20 points. Group I patients (5 to 7 points) showed a maximal increase of E-$\alpha_1$PI up to 5-fold above normal values and group II patients (9 to 11 points) up to 10-fold. In group III (14 to 17 points) peak levels higher than 20-fold above normal were measured. During the further observation period (up to 100 h) a significant decrease of E-$\alpha_1$PI plasma levels towards normal values was observed in all patients.

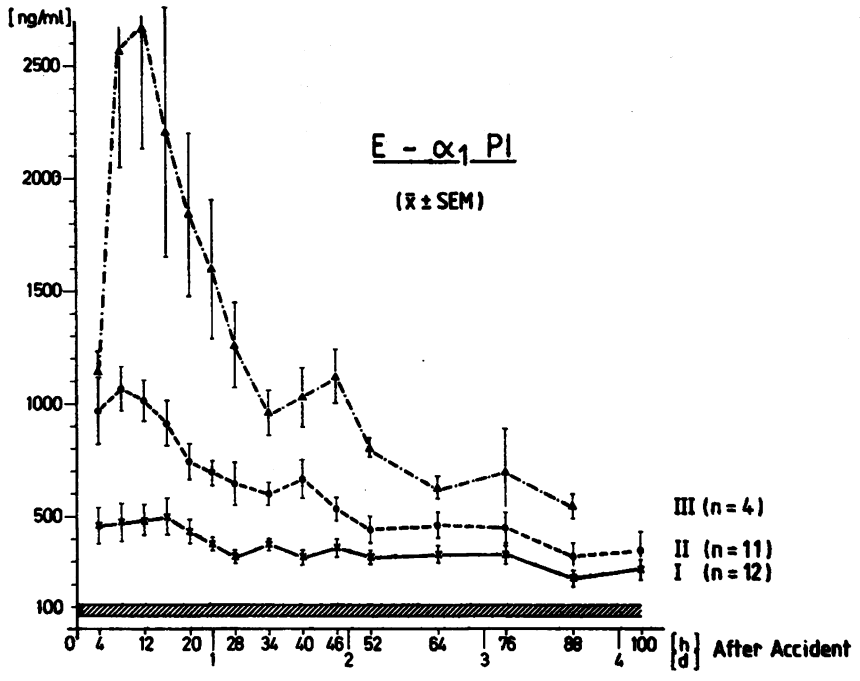


Fig. 10. Plasma levels of E-$\alpha_1$PI complex in patients after multiple trauma. On the basis of a hospital internal scale with 20 points (HISP) patients were allied to 3 groups reflecting the severity of injury (I milde; II severe; III highly severe):

| Group | patients (n) | HISP |
|---|---|---|
| I | 12 | 6.3 ± 0.6 |
| II | 11 | 10.0 ± 1.0 |
| III | 4 | 15.3 ± 1.0 |

Normal range of E-$\alpha_1$PI: 60 - 110 ng/ml

Surprisingly, no correlation between the amount of complexed elastase and other plasma factors, e. g. antithrombin III, factor XIII, prothrombin, $\alpha_2$-macroglobulin, plasminogen, and $\alpha_2$-antiplasmin could be demonstrated so far.

To see, whether this might be due to massive blood transfusions in polytrauma patients, we measured the activities of several plasma proteins in stored blood as a function of the storage period. The activities of the plasma proteins antithrombin III, prothrombin and $\alpha_2$-macroglobulin were in the range of normal freshly drawn blood and stayed quite stable throughout the storage period, whereas the complexed elastase increased considerably up to more than 60-fold of normal within 35 days (data not shown). From these observations and the results seen in polytrauma patients we draw two conclusions:

(i) Due to massive blood transfusions and relatively long half life periods of the blood proteins probably also in the circulation, the concentration of these factors in patients'plasma are nearly not influenced by the early endogenous liberation of granulocytic elastase after polytrauma.

(ii) High levels of complexed elastase in patients' plasma might be also caused by blood transfusions.

However, in a more detailed investigation comparison of the E-$\alpha_1$PI levels in patients' plasma with hypothetic levels - considering both: the amount of complexed elastase administered with the transfused blood and the elimination of the complex from the circulation ($t_{1/2}$ of approx. 1 h) - showed no coincidence at all. Probably, in patients receiving massive blood transfusions a relationship between neutrophil elastase release and consumption of humoral factors is measurable only locally, e. g. in lung lavage fluid or blood samples of the wounded or inflamed area. Nevertheless, the high E-$\alpha_1$PI levels occuring in severely injured patients are a clear indication of a strong inflammatory response ongoing in the organism.

## CONCLUSIONS

In severe inflammatory processes,multiple trauma or shock various cells like neutrophils, macrophages, endothelial cells and mast cells are stimulated or disintegrated. In this way a high

potential of lysosomal enzymes is released of which the proteinases are of special pathogenic effectiveness. Recent studies in our laboratory and by others (Egbring et al., 1977) indicate strongly that substrate-unspecific proteolysis by lysosomal proteinases, especially by the neutrophil elastase, contributes to a significant degree to the consumption and/or degradation of extracellular substances in such diseases. The amount of the E-$\alpha_1$PI complex reflects the intensity of both the inflammatory stimulus and the response of the neutrophils. Hence, in most cases an increase of the E-$\alpha_1$PI level in the circulation represents a systemic signal of a local inflammatory event. Therefore, the clinical situation of the patient has to be carefully considered for diagnostic interpretation of E-$\alpha_1$PI levels in plasma. Nevertheless, to avoid a deleterious endogenous proteinase-proteinase inhibitor imbalance, early administration of convenient exogenous inhibitors directed against lysosomal enzymes should have a positive therapeutic effect also in humans.

## ACKNOWLEDGEMENTS

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## DISCUSSION

ELŐDI, S.:

Did you measure the amount of AT-III in septic patients? If the amount of antigen is in the normal range, one could differentiate whether the decreased activity is either due to consumption or to proteolytic damage on the inhibitor, since AT-III modified by proteolysis may still hold antigenic properties.

JOCHUM:

Unfortunately, immunological methods such as one- or two-dimensional immunoelectrophoresis can not differentiate between antithrombin III complexed with thrombin and antithrombin III degraded by elastase. We have done these experiments several years ago.

BOHLEY:

I wonder in what kind of lysosomes metallo-proteinases may occur.

JOCHUM:

It was reported that macrophages contain an elastase-like proteinase which was identified as a metallo-proteinase.