

## Cathepsin B – Indicator for the Release of Lysosomal Cysteine Proteinases in Severe Trauma and Inflammation

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### Introduction

Lysosomal elastase from polymorphonuclear (PMN) granulocytes is believed to be an important nonspecific mediator of inflammation contributing to the development of sepsis- and trauma-related organ failure [1]. We have found previously that also the lysosomal cysteine proteinase (CP) cathepsin B or a cathepsin B-like enzyme is released into the blood plasma of patients during septic shock [2]. In contrast to PMN elastase, released cathepsin B can be detected by its enzymatic activity because it is only loosely bound to the plasma inhibitors and dissociates readily upon dilution.

Here we want to report further evidence for the release of cysteine proteinases into the circulation of polytraumatized patients and, locally, into the alveolar epithelial lining fluid and purulent peritoneal exudates. Increased cysteine proteinase activity was also observed in the blood plasma of pigs subjected to experimental septic shock. In all cases the released cysteine proteinase activity was identified as that of cathepsin B or a cathepsin B-like enzyme. Its interaction with the plasma cysteine proteinase inhibitors was investigated in vitro using samples from individual patients. Indirect evidence will be provided that the main portion of the released lysosomal cysteine proteinase(s) should originate from cells others than PMN granulocytes, most probably from macrophages, and may play a role as nonspecific mediator(s) of inflammation.

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#### *Enzymes:*

Cathepsin B (EC 3.4.22.1); Cathepsin H (3.4.22.16); Cathepsin L (3.4.22.15); Leukocyte elastase (EC 3.4.21.37).

#### *Abbreviations:*

$\alpha_2$ M,  $\alpha_2$ -macroglobulin;  $\alpha_1$ PI,  $\alpha_1$ -proteinase inhibitor; ARDS, adult respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CP, cysteine proteinase(s); ELISA, enzyme-linked immunosorbent assay; E-64, 1-(*trans*-epoxysuccinyl-L-leucylamino)-4-guanidinobutane; FPLC, fast protein liquid chromatography; NMec, (4-methyl-7-coumaryl)amide; PMN, polymorphonuclear; SEM, standard error of the mean; Z, benzyloxycarbonyl.

## Materials and Methods

### *Patients and samples*

Details about the organization of the clinical studies and sampling protocols are provided in ref. [3] for polytraumatized patients, in ref. [4] for BALF and in ref. [5,6] for peritonitis exudates. Experimental septic shock in anesthetized pigs was performed as described [7]. All samples were stored frozen and thawed only once immediately before determination.

### *Separation and lysis of blood cells*

Heparinized human blood, diluted with tissue culture medium RPMI 1640 (Gibco) was layered over lymphocyte separation medium MSL ( $d=1.077$ , Eurobio) and centrifuged. The mononuclear cells were recentrifuged in RPMI and lysed in 50 mM sodium acetate buffer pH 5.5 containing 0.5 % Triton X-100, 0.3 mM EDTA and 0.01 % Brij 35. For preparation of PMN granulocytes Mono-Poly Resolving Medium (Flow) was used.

### *Assay for cysteine proteinase activity*

CP activity was measured in continuous assays with the fluorogenic substrates Z-Phe-Arg-NMec, Z-Arg-Arg-NMec and Arg-NMec using the specific inhibitor E-64 as described [2].

### *Stopped assay for papain inhibiting capacity*

Increasing volumes of sample dilutions were incubated with papain (7 nM) in acetate buffer pH 5.5, 1 mM dithiothreitol, 2 mM EDTA, 0.015 % Brij 35, for 25 min at 30 °C. After dilution the substrate Z-Phe-Arg-NMec (10  $\mu$ M) was added and the reaction was stopped with monochloroacetate after 15-25 min.

### *ELISA for human cathepsin B*

Immunoselected polyclonal antibodies against human cathepsin B were prepared from sheep antiserum with cathepsin B immobilized on CNBr-activated Sepharose. Cathepsin B bound to microtiter plates coated with the antibodies was determined in a sandwich ELISA using biotinylated antibodies and biotin-avidin-peroxidase for detection.

### *ELISA for human elastase*

Granulocytic elastase was estimated in complex with  $\alpha_1$ -proteinase inhibitor according to ref. [5] using a specific two-site ELISA.

### *Gel chromatography*

Separation of patient plasma on a Superose 12 FPLC column was performed as described [2]. Inhibitory activity of the individual fractions was determined in stopped assays as above using 1/40 of each fraction (0.4 ml) with 200 pM papain.

## Results

### CP activity in blood plasma of polytraumatized patients

CP activity of blood plasma was followed in 69 intensive care patients due to severe trauma with a mean injury severity score (ISS) according to Baker et al. [8] of 36 (see [3] for details). Within 6 h after the injury, increased levels of CP activity (mean: 5 - 6-fold of normal) were found in the blood plasma of patients who developed lethal (Fig. 1 A) or reversible (Fig. 1 B) multiple organ failure several days later. Initial CP activity was significantly lower (mean: 3-fold of normal) in polytraumatized patients without subsequent organ failure (Fig. 1 C). In all three groups CP activity reached its maximum 6 h before  $\alpha_1$ PI-bound PMN elastase, and returned to values about 2-fold of the normal within 3 days.

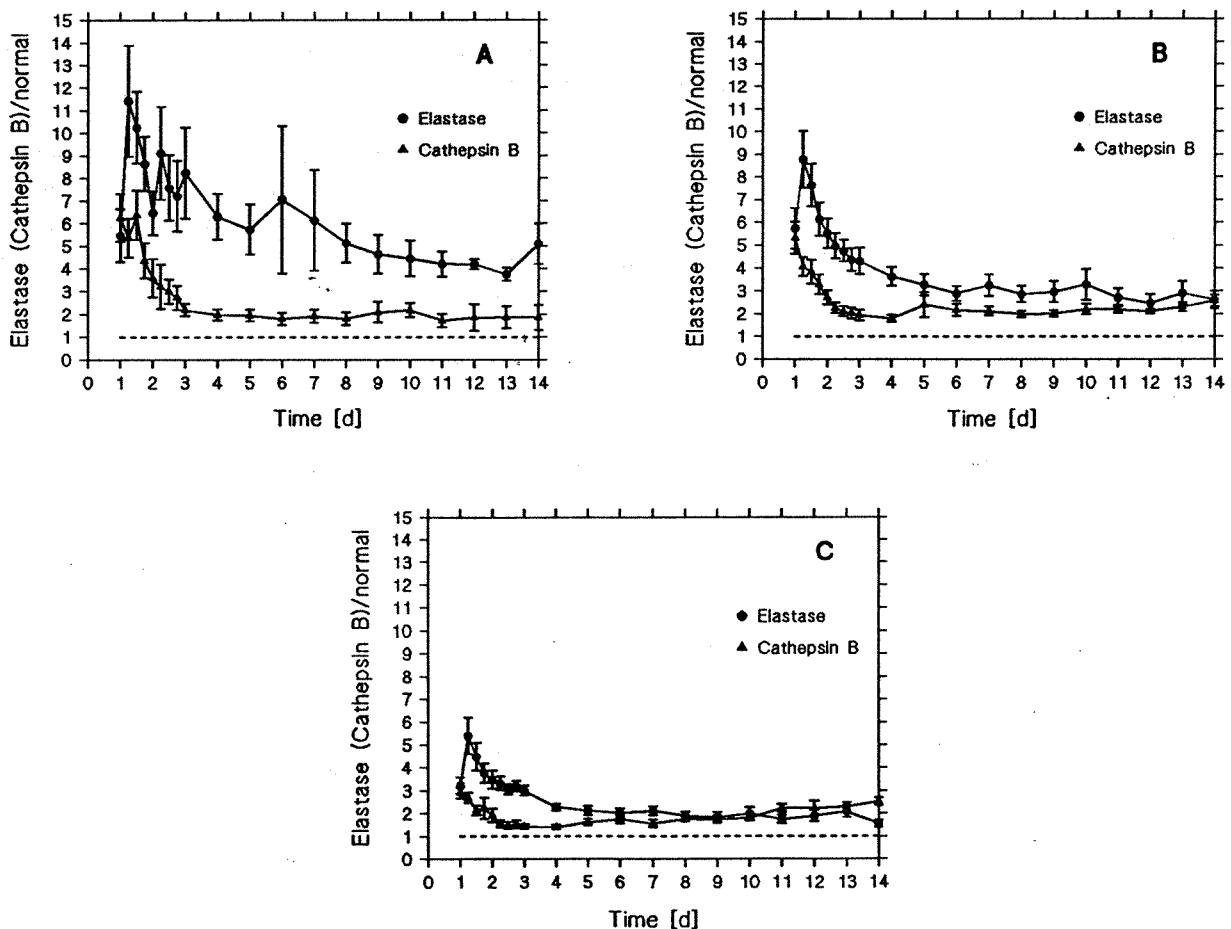


Fig. 1. Cathepsin B activity and  $\alpha_1$ PI-bound elastase levels in blood plasma of polytraumatized patients expressed as the multiple of normal values (50 mU/l for cathepsin B, 90 ng/ml for elastase). A, patients with lethal organ failure (n=11); B, patients with reversible organ failure (n=29); C, patients without organ complications (n=29).

As reported previously [2], dilution experiments revealed that the CP-activity of blood plasma is in equilibrium with reversible cysteine proteinase inhibitors. Evaluation of dilution experiments performed with plasma of individual polytraumatized patients (Fig. 2) provided estimates of  $K_m = 98 \mu\text{M}$  and  $I_t/K_i = 15\text{--}20$  ( $K_m$ , Michaelis constant;  $I_t$ , total inhibitor concentration;  $K_i$ , inhibition constant). The routine assay used in the clinical studies with polytraumatized patients (80  $\mu\text{l}$  Plasma in 1.5 ml total volume, 150  $\mu\text{M}$  substrate) detects approx. 60 % of total CP-activity as free, active enzyme.

### CP activity in bronchoalveolar lavage fluid

One of the fatal complications of polytraumatized patients is the adult respiratory distress syndrome (ARDS). We found that bronchoalveolar lavage fluid (BALF) of a patient with ARDS contained high levels of CP activity compared to that of a polytraumatized patient without this complication (Fig. 3). For comparison with PMN elastase, the CP activity was converted into ng/ml cathepsin B assuming a specific activity of 80 U/mg for purified cathepsin B [2]. The maximal levels of  $\alpha_1\text{PI}$ -bound elastase were approx. 20-fold higher than those of cathepsin B and did not always coincide with the latter. In some samples an elastase/cathepsin B ratio close to 1.0 was observed.

Cathepsin B concentrations measured with an ELISA using specific antibodies against human lysosomal cathepsin B correlated well with the concentrations calculated from activity (Table 1). These results indicate that, in this patient, virtually all measured cysteine proteinase activity was due to cathepsin B (or a cross-reactive proenzyme) and most of the immunologically detectable enzyme was enzymatically active. Titration of papain with BALF revealed the absence of cysteine proteinase inhibitors (less than 10 nM compared to 5  $\mu\text{M}$  in normal plasma) and no dissociation was observed in dilution experiments.

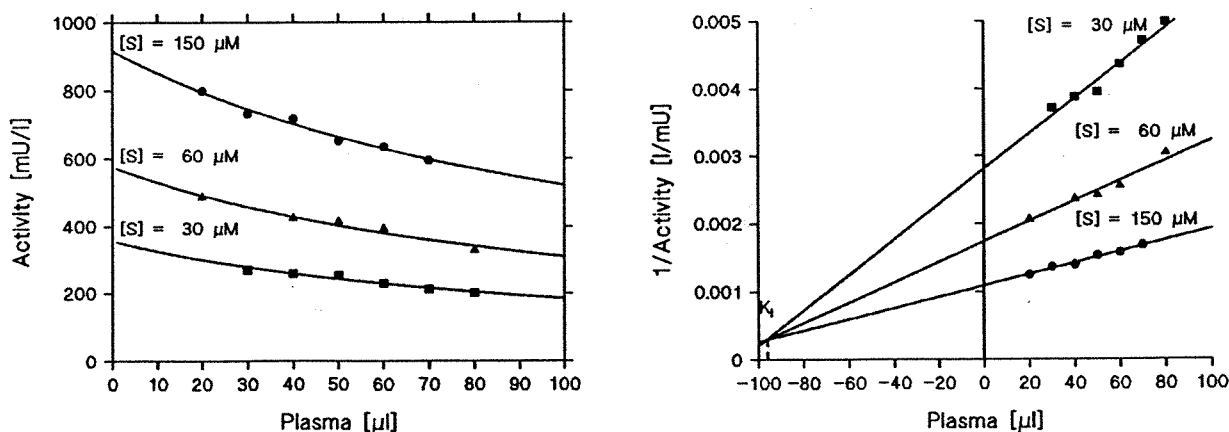


Fig. 2. Dilution analysis of CP activity in blood plasma of a polytraumatized patient. From a replot according to Dixon [9] (lower panel)  $K_m$  was estimated as 98  $\mu\text{M}$  and a  $I_t/K_i$  of 16 was calculated for competitive inhibition ( $K_m$ , Michaelis constant;  $I_t$ , total inhibitor concentration;  $K_i$ , inhibition constant).

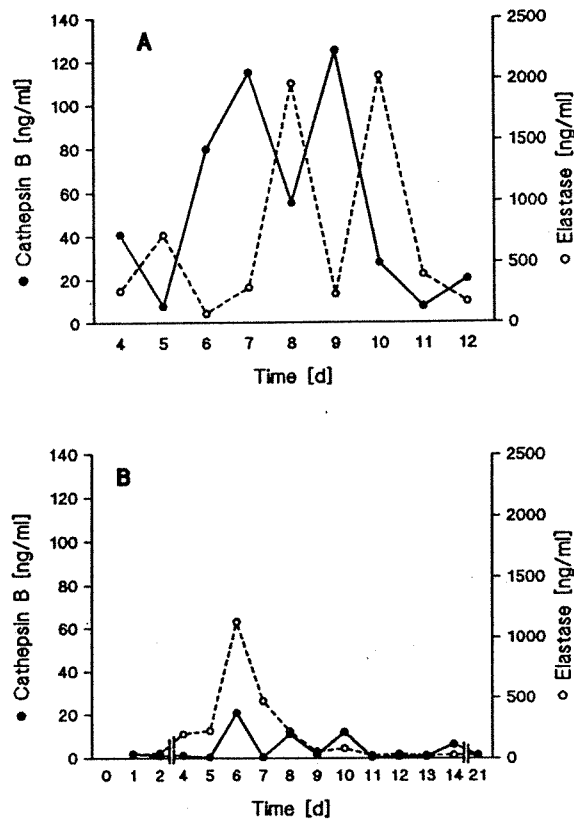


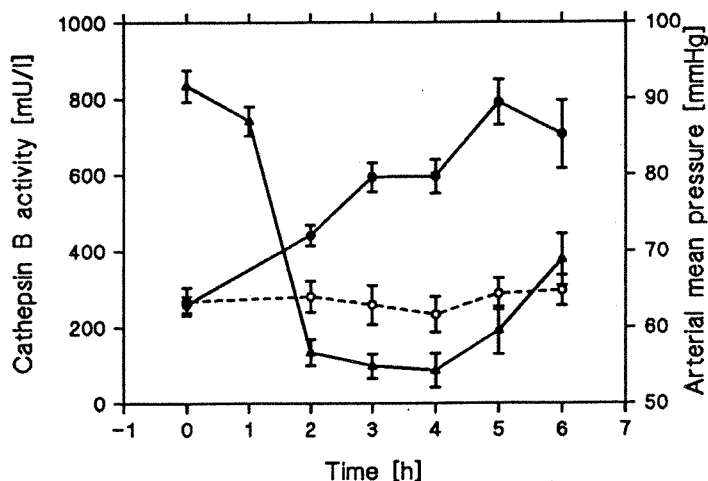
Fig. 3. Levels of cathepsin B (filled circles) and  $\alpha_1$ PI-bound elastase (open circles) in bronchoalveolar lavage fluid of polytraumatized patients with ARDS (A) and without ARDS (B). The cathepsin B level was calculated from enzymatic activity assuming a specific activity of 80 U/mg.

Table 1. Correlation between CP activity and immuno-reactive cathepsin B (ELISA) in BALF of a patient with ARDS

Sample <sup>a</sup>	CP activity [mU/l]	Enzymatically active cathepsin B <sup>b</sup> [ng/ml]	Immunoreactive cathepsin B [ng/ml]
431/1	3 267	40.8	39.7
431/5	618	7.7	8.0
431/7	9 194	114.9	121.7
431/8	4 429	55.4	46.6
431/10	2 232	27.9	23.6
431/11	618	7.7	10.8
431/12	1 622	20.3	26.2

<sup>a</sup>see Fig. 3 for comparison

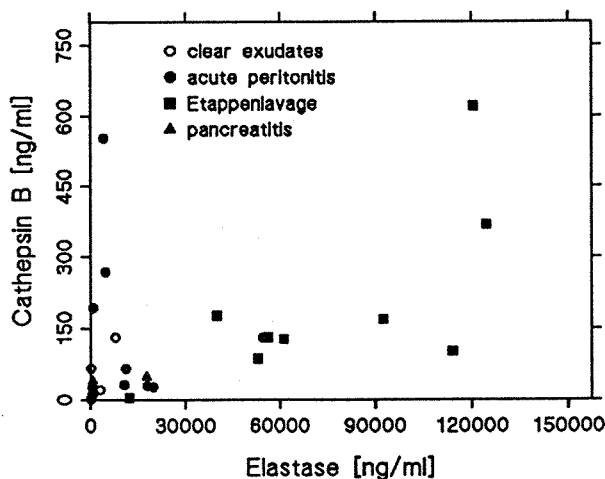
<sup>b</sup>calculated assuming a specific activity of 80 U/mg



**Fig. 4.** Cathepsin B activity in blood plasma of pigs subjected to experimental endotoxin shock. 19 anesthetized miniature pigs received an i.v. infusion of bacterial lipopolysaccharide from *S. abortus equi* for 6 hours and developed a severe septic shock with low arterial mean pressure (solid triangles). Cathepsin B activity increased (solid circles) as compared to control animals (n=7) receiving an NaCl infusion (open circles). Data are mean values  $\pm$  SEM.

#### CP activity in experimental endotoxin shock of pigs

A reproducible increase of CP activity of blood plasma was observed in pigs subjected to severe septic shock by infusion of bacterial endotoxin. Cathepsin B activity correlated with physiological shock parameters like decrease of arterial mean pressure (Fig. 4).



**Fig. 5.** Cathepsin B versus  $\alpha_1$ PI-bound elastase levels in peritoneal exudates. Cathepsin B levels were calculated from enzymatic activity assuming a specific activity of 80 U/mg;  $\alpha_1$ PI-bound elastase was determined by ELISA [5]. Open circles, clear exudates (n=9); solid circles, acute peritonitis (n=9); solid squares, persistent peritonitis treated by Etappenlavage (n=9); solid triangles, pancreatitis (n=2).

### CP activity in peritoneal exudates

Peritonitis exudates contain high levels of  $\alpha_1$ PI-bound and even free elastase which is believed to contribute to nonspecific proteolytic breakdown of opsonins like C3 and IgG resulting in diminished opsonizing activity [5]. We found also high levels of CP activity in most of these exudates (Fig. 5, Table 2). CP activity did not always parallel  $\alpha_1$ PI-bound elastase. High CP activity and moderate elastase levels were found in cases of acute peritonitis whereas both CP activity and elastase concentration tended to be extremely elevated in patients with persistent purulent peritonitis treated by Etappenlavage [6]. CP activity and elastase were low in clear exudates.

Like in plasma, CP activity in peritoneal exudates dissociates reversibly from complexes upon dilution. Typical  $K_m$  and  $I_t/K_i$  were determined as 150  $\mu$ M and 50, respectively.

**Table 2. Elastase and cathepsin B in peritoneal exudates**

Samples	Elastase mean $\pm$ SEM [ng/ml]	Cathepsin B mean $\pm$ SEM [ng/ml <sup>a</sup> ]	Elastase/ cathepsin B
Clear exudates (n = 9)	1 502 $\pm$ 848	30 $\pm$ 14	49
Acute peritonitis (n = 9)	13 809 $\pm$ 5 584	146 $\pm$ 58	95
Etappenlavage (n = 9)	74 900 $\pm$ 13 219	198 $\pm$ 62	378

<sup>a</sup>calculated from enzymatic activity assuming a specific activity of 80 U/mg

### CP activity of blood monocytes and granulocytes

Lysed isolated granulocytes (98 % of total cells) from normal peripheral blood were found to have a low but significant CP activity of 115 mU/10<sup>6</sup> cells corresponding to 1.4 ng/10<sup>6</sup> cells of cathepsin B. 10-20-fold higher CP activity was found in lysates of a fraction containing 14.5 % monocytes, 83.9 % lymphocytes and only 0.16 % granulocytes.

### Substrate specificity of CP activity

The specificity of CP activity in blood plasma, BALF and peritoneal exudates for small synthetic peptide substrates closely resembles that of isolated human cathepsin B (Table 3). As judged from activity with the aminopeptidase substrate Arg-NMec, less than 1 % of the endopeptidase activity with Z-Phe-Arg-NMec can be attributed to cathepsin H. Because of the low specific activity of cathepsin H (1.5 U/mg as calculated from  $k_{cat}$  and  $K_m$  in ref. [11],

its presence in appreciable amounts cannot be excluded. A significant contribution of cathepsin L (specific activity 36 U/mg with Z-Phe-Arg-NMec according to ref. [12], however, is ruled out by the estimated  $K_m$  values (see Table 3) and the observed  $I_t/K_1$  (see Discussion).

**Table 3. Substrate specificity of CP activity**

Sample	Ratio of activities with		$K_m$ ( $\mu M$ ) <sup>a</sup>
	Z-Arg-Arg-NMec/ Z-Phe-Arg-NMec [145 $\mu M$ ]	R-NMec/ Z-Phe-Arg-NMec [56 $\mu M$ ]	
Isolated lysosomal cathepsin H		17	
cathepsin L	< 0.0002		2.4 <sup>b</sup>
cathepsin B	0.22	< 0.00005	160 <sup>c</sup>
Human monocytes <sup>d</sup>	0.20	n.d.	n.d.
Human granulocytes <sup>d</sup>	0.19	n.d.	n.d.
Peritonitis exudate	0.19	0.014	150
BALF (patient with ARDS)	0.12	0.006	225
Plasma (polytrauma)	0.07	0.04	95
Plasma (sepsis)	0.22	0.08	170 <sup>c</sup>

<sup>a</sup>with Z-Phe-Arg-NMec, determined from dilution experiments unless otherwise indicated

<sup>b</sup>from ref. [12]

<sup>c</sup>from ref.[2]

<sup>d</sup>from lysates of isolated cells of normal peripheral blood

### Endogenous inhibitors of cysteine proteinases

The inhibitory capacity for CP was determined by titration of a constant amount of papain with increasing amounts of samples (plasma, exudate, BALF) from individual patients. Equivalence was assumed when a constant enzymatic activity was reached. This constant activity is due to papain entrapped in  $\alpha_2M$  where it remains still active against the small synthetic substrate [13]. The remaining activity (10-40%) was subtracted from the initial activity of papain. The papain inhibiting capacity of normal blood plasma is around 5  $\mu M$ . Reduced inhibitory capacity was found in plasma samples of some polytraumatized patients. BALF contained less than 10 nM free inhibitors (Table 4).

Chromatography of plasma from a polytraumatized patient on a Superose 12 FPLC column separated two main peaks of inhibitory activity corresponding to molecular masses of 70 kDa and 15 kDa, respectively (data not shown). Weak inhibition of papain activity was also found in the high molecular mass region where  $\alpha_2M$  is eluted.



**Table 4. Inhibitory capacity for papain**

Sample	Inhibitory capacity for papain [ $\mu\text{mol/l}$ ]	Non-titrable activity [% of total]
Normal plasma (n=2)	4.2 - 5.6	23 - 29
Polytrauma plasma (n=15)	1.4 - 5.6	14 - 26
Peritoneal exudate (n=6)	0.6 - 2.5	9 - 14
BALF (n=3)	< 0.01	n.d.

## Discussion

### Nature and cellular origin of released CP activity

The presented results of a clinical study with polytraumatized patients confirmed and extended previous observations that, besides the lysosomal serine proteinases PMN elastase and cathepsin G, also cysteine proteinases are released into the circulation under conditions of severe trauma and inflammation. High levels of this enzymatic activity were detected locally in bronchoalveolar lavage fluid and peritoneal exudates. As in previous work, the specificity of our assay for this class of proteinases was ensured by the use of the specific active-site inactivator E-64 [14]. Activity measurements using different peptide substrates and  $K_m$  determinations from Dixon plots (see Table 3) indicated that the enzymatic activity of all studied samples should be mainly due to cathepsin B or a cathepsin B-like enzyme. In the case of BALF this was directly confirmed with a specific ELISA for human cathepsin B.

Though Z-Arg-Arg-NMec would be more specific for cathepsin B (cf. Table 3), Z-Phe-Arg-NMec has been selected for routine assay, because this substrate is 4 times more sensitive for cathepsin B-like activity and less sensitive to E-64-resistant non-cysteine proteinase activity found in some samples [2].

In blood plasma as well as in local secretions the observed cathepsin B-like activity correlates with the severeness of the clinical manifestation of organ dysfunctions. High cathepsin B activity of blood plasma detectable soon after the traumatic event seems to be a sensitive and specific parameter for the prediction of trauma-related organ failure (see ref. [3] for details).

As we have confirmed in this work, PMN granulocytes of normal blood contain only minor amounts of cathepsin B ( $1.5 \text{ ng}/10^6$  cells), compared to  $5-9 \mu\text{g}/10^6$  cells of elastase [15]. The ratio of cellular elastase/cathepsin B content (3000-6000) is roughly 10-30-fold higher than the ratio of released elastase/cathepsin B in blood plasma of polytrauma patients (200-300) and in peritonitis exudates (100-400). Therefore simultaneous release of both enzymes from PMN granulocytes can probably not account for the observed

cathepsin B levels. Measurement of intracellular cathepsin B activity by flow cytometry indicated, however, that PMN granulocytes may increase their cathepsin B content under inflammatory conditions (G. Rothe and G. Valet, unpublished results). Moreover, in peritoneal exudates, a contribution of bacterial cysteine proteinases has to be considered.

An elastase/cathepsin B ratio close to 1.0 was found in BALF which is known to contain large numbers of alveolar macrophages [16]. According to the cellular contents [17,18], macrophages must be considered as a main source for released cathepsin B. In cell lysates, monocytes of normal peripheral blood contain 10–20 times more cathepsin B activity than granulocytes. Recently, a sensitive flow cytometric assay for intracellular cathepsin B activity has been developed (G. Rothe et al., unpublished). Using this assay, a 55-fold higher cathepsin B activity was detected in living human monocytes as compared to neutrophils. The 5-fold higher cytometric response of thioglycolate-elicited rat peritoneal macrophages in comparison to resident peritoneal macrophages suggests that cathepsin B activity is indeed a cellular marker for activation and differentiation of macrophages. In blood plasma and secretions, increased cathepsin B activity accompanied by an decreased elastase/cathepsin B ratio may turn out to be an extracellular plasmatic marker for macrophage activation.

#### Interaction of CP activity with endogenous protein inhibitors

Papain, a plant cysteine proteinase, was used to determine the inhibitory capacity for cysteine proteinases, because this enzyme is very similar to lysosomal cathepsin L. Both proteinases bind tightly to all known cysteine proteinase inhibitors of blood and tissues [19]. According to the results of the papain assay, blood plasma and peritoneal exudate contain a high inhibitory capacity for cysteine proteinases. The inhibitory capacity of blood plasma was found to be reduced to about 30 % in some polytrauma patients. It returns to normal or slightly reduced values within two weeks.

Dixon plot analysis of plasma samples from individual patients (see Fig. 2) provided estimates of the ratio  $I_t/K_i$ . A ratio of 20 found in plasma of polytraumatized patients points to the kininogens (expected  $I_t/K_i = 8-160$ ) and cystatin C (expected  $I_t/K_i = 50-120$ ) as the most important inhibitors involved in the equilibrium with cathepsin B [2]. This is consistent with the molecular masses of the observed inhibitory fractions in blood plasma. Cathepsin L is bound to kininogen and cystatin C much more tightly than cathepsin B and would not be detectable as active enzyme ( $I_t/K_i$  of  $3 \times 10^5$  with kininogen and  $2 \times 10^4$  with cystatin C). Surprisingly, only traces of cathepsin B activity have been found associated with  $\alpha_2M$  whereas papain is entrapped readily [2].

#### Pathobiochemical relevance of cathepsin B

It is tempting to speculate that increased cathepsin B-like activity is directly involved in pathomechanisms leading to trauma-related organ failure, such as ARDS, or persistent peritonitis. As far as data are available, isolated cathepsin B is not very active on protein substrates [20], but this may be

different for cathepsin B precursors which have been found in sputum [21] and in tumors [22]. The proteolytic potential of the released cathepsin B-like activity for the cleavage of physiologically important proteins needs to be investigated in more detail. Besides its own proteolytic activity, cathepsin B may be envisaged as an indicator for the simultaneous release of other cysteine proteinases. Unlike cathepsin B, cathepsin L has been shown to cleave elastin [23] and to inactivate  $\alpha_1$ PI [24]. The decreased inhibitory capacity observed in blood plasma of patients and in BALF may enhance the harmful effects of released lysosomal cysteine proteinases.

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