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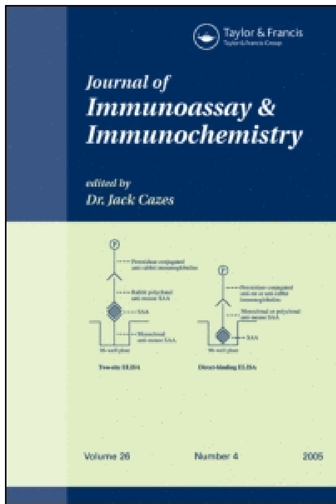

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Establishment of an Enzyme-Linked Immuno-Sorbent Assay for Urinary Trypsin Inhibitor by Using a Monoclonal Antibody

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JOURNAL OF IMMUNOASSAY, 12(3), 347-369 (1991)

ESTABLISHMENT OF AN ENZYME-LINKED IMMUNO-SORBENT ASSAY FOR URINARY TRYPSIN INHIBITOR BY USING A MONOCLONAL ANTIBODY

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ABSTRACT

Monoclonal antibodies against inter-α-trypsin-inhibitor (ITI) were produced. One clone showing specificity for urinary trypsin inhibitor (UTI), a proteolytic fragment of ITI, which is excreted into urine, was selected for the establishment of an enzyme-linked immuno-sorbent assay (ELISA). The ELISA for the quantification of UTI was shown to work reproducibly in the range between 0.5 and 10 ng UTI/ml urine. Urines of several patients suffering from different lung diseases were screened for UTI using the established ELISA. Highest UTI levels were found in the urine of patients with lung empyema. A more moderate increase was observed in patients suffering from lung tuberculosis and from secondary and primary lung tumors.
(KEY WORDS: enzyme-linked, proteinase inhibitor, lung-disease)

INTRODUCTION

Inter-α-trypsin-inhibitor (ITI) is a high molecular mass glycoprotein (about 240 kDa) present in human

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plasma at a concentration of 0.4 g/l. It was first isolated in 1962; its function as a proteinase inhibitor, however, was established not before 1965 (1,2, for review see 3). When ITI is exposed to proteolytic enzymes (e.g. pancreatic trypsin or elastase), a low molecular mass compound with antiproteolytic activity, termed HI-30 appears in the reaction mixture. The molecular mass of HI-30 varies in the range between 30 and 60 kDa. Amino acid sequencing of HI-30 (4) revealed that it is identical to the acid-soluble serum-trypsin-inhibitor (STI), which appears in urine after its renal filtration and is therefore also named urinary-trypsin-inhibitor (UTI). UTI and STI have identical amino-acid sequences, however, they may be different in their carbohydrate composition. In this context acid soluble means that in contrast to intact ITI the low molecular weight components could not be precipitated by treatment with perchloric acid. Description of these low molecular weight ITI-related proteinase inhibitors led to the concept that in extracellular fluids ITI acts as a "precursor" molecule from which low molecular weight trypsin inhibitory components are proteolyticaly liberated (3,5). However, the concept of the precursor-effector relationship has recently been modified by Gebhard et al.: according to their findings ITI is a complex of three distinct protein subunits rather than

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a single-chain protein (6,7). The inhibitory subunit shows a molecular weight of 56 kDa and is combined in the complex with two other consituents of 78 kDa and 85 kDa. The detailed structure of the ITI-complex remains a matter of debate. In any case it is appropriate to speak of ITI-subunits or ITI-related peptides rather than of ITI-"fragments". UTI contains two Kunitz-type inhibitory domains, the N-terminal domain responsible for inhibition of elastase and the C-terminal domain which inhibits trypsin activity (8). Based on this fact "bikuin" has recently been suggested as a common name for UTI and STI (HI-30) (9).

By complex biochemical procedures increased levels of UTI in urine were detected during severe inflammation (10) as well as in cancer (11). Moreover, increased levels of the inhibitor were found in bronchial secretions during obstructive lung disease (12). Under these conditions the inhibitor is thought to be involved in the control of extracellular proteolysis especially in counteracting proteolytic enzymes derived from leukocytes of the inflammatory cellular infiltrate (13). However, the routine investigation of patient samples was hampered by the lack of a convenient and reliable assay system for UTI.

We established a series of monoclonal antibodies recognizing distinct epitopes of the ITI molecule. One of

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these mAbs (IATI5) recognizing an epitope located at the HI-30 peptide was used to set up an enzyme-linked immuno-sorbent assay (ELISA) for the quantification of acid-soluble UTI. Using this assay urines from patients suffering from different lung diseases were investigated for their UTI content. This preliminary study shows, that increased levels of UTI were present in urine of patients with inflammatory and malignant lung diseases. Highest levels were found in urine of patients suffering from lung empyema. However, the role of UTI in lung disease remains to be explored.

MATERIALS AND METHODS

Monoclonal and polyclonal Antibodies

Female BALB/c mice (5-10 weeks old; Charles River Wiga GmbH, Sulzfeld, FRG) were immunized with a conventionally purified preparation of high molecular weight ITI from human plasma kindly provided by Dr. Heimburger (Behringwerke AG, Marburg, FRG). Hybridoma production was performed as described in (14). Monoclonal antibodies were tested for their reactivity with ITI and ITI related peptides including HI-30.

A polyclonal immunoglobulin preparation was obtained by immunizing outbred white rabbits with HI-30. HI-30 was prepared from intact ITI as described in (15).

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Patients and Collection of Urine Samples

Morning urine was collected from patients suffering from lung empyema (n = 9), lung tuberculosis (n = 9), primary and secondary lung tumors (n = 22) as well as from healthy persons (n = 13). Two urine samples were taken from each patient at an interval of 2 days. After determination of urinary creatinine, aliquots were frozen at - 20 °C until UTI was determined by ELISA.

Electrophoresis and Immunoblotting

Electrophoresis and immunoblotting were performed in order to characterize the specificity of the monoclonal antibody IATI5.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in gradient gels (5 - 20 % or 10 - 20 %, respectively) under reducing conditions according to Laemmli (16). Usually 1-5 µg protein/lane was applied to the gel. After electrophoresis the proteins were visualized by silver staining as described by Oakley et al. (17). Urinary proteins were dialysed against water and concentrated 5 times by lyophilisation.

Immuno-blotting was performed according to Khyse-Andersen (18). UTI was visualized by using the mAb IATI5 as a primary antibody followed by colloidal gold staining according to Moeremans et al. (19). Briefly,

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after blotting nitrocellulose sheets were incubated in 5 % (w/v) bovine serum albumin (BSA) in Tris buffer (0,02 mol/l, pH 8,2, containing 0,9 % (w/v) NaCl) for 30 minutes in order to block non-specific binding sites. The sheets were then incubated overnight in a solution of monoclonal antibody IATI5 (0.2 µg/ml Tris buffer containing 0,1 % (w/v) BSA). After washing the sheets were treated with anti-mouse IgG immunoglobulin G from rabbit (Behring, Marburg, FRG), which was used in a 1 : 1000 dilution in Tris buffer/0,1 % (w/v) BSA. Proteins recognized by IATI5 were visualized using gold labeled goat anti rabbit IgG antibodies (Janssen, Olen, Belgium). Positive gold labeling was enhanced by silver staining (silver enhancement kit, Janssen, Olen, Belgium).

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Quantification of acid-soluble UTI by Enzyme-linked Immuno-sorbent Assay

UTI, the acid-soluble trypsin-inhibitory subunit of ITI in urine was quantified in a solid phase enzyme-linked immuno-sorbent assay. Rabbit anti-HI-30 immunoglobulin G bound to microtiter plates (Nunc, Roskilde, Denmark) served as catching reagent. Plates were washed 3 times with PBS containing 0,1 % (v/v) Tween and 1 % (w/v) BSA after each incubation step.

Deproteination and enrichment of acid-soluble compounds in the test sample was achieved by perchloric acid

treatment: 100 µl of the test sample were added to 500 µl of perchloric acid (0.6 mol/l; no. 125 377, Boehringer Mannheim, FRG) and mixed by repeated pipetting. After centrifugation (2 x 2 min, 10,000 x g), 400 µl of the clear supernatant were removed and neutralized by addition of 50 µl of potassium carbonate (2.5 mol/l; no. 4928, Merck AG, Darmstadt, FRG). The mixture was spun again (10,000 x g, 2 x 2 min) and the clear supernatant diluted 1:10, 1:100, and 1:500 in PBS/Tween/ 1 % (w/v) BSA was taken for analysis in enzyme immuno assays. Native urine was used for the determination of UTI by ELISA. For calibration of the assay a pure preparation of HI-30, produced from plasma-ITI by trypsin-treatment and further purified by gelfiltration, was tested in graded dilutions. A 1:5000 dilution of monoclonal antibody IATI5 (0.2 µg/ml PBS/Tween/0,1 % (w/v) BSA) was then added for 1 h. Afterwards rabbit-anti-mouse IgG-peroxidase conjugate (Dianova, Hamburg, FRG) was added and incubated for 1 h. Bound peroxidase labeled antibody was quantified by using the peroxidase-substrate H_2O_2/o-phenylendiamine and photometric measurement of the reaction product at 492 nm.

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Quantification of α_1-Proteinase-Inhibitor-Elastase Complexes

α_1-proteinase-inhibitor-(α_1PI-)elastase complexes were determined in citrated plasma (stored at -20 ^{o}C) by

using a commercially available test kit (PMN Elastase IMAC, order no. 11332; Merck, Darmstadt, FRG) in combination with an analyser (EPOS, Eppendorf, Hamburg, FRG). Plasma samples were taken at the same day as the urine samples from the respective patients.

Creatinine Determination

Creatinine was determined immediately after collection of urine samples by using a commercially available test kit (Merckotest no. 3385; Merck, Darmstadt, FRG) in combination with an analyser (EPOS, Eppendorf, Hamburg, FRG).

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Protein Determination

Protein was determined according to Bradford (20) using bovine serum albumin as a standard.

Regression Analysis and Statistics

Linear regression analysis was performed using a microcomputer based statistical program. Pearson's correlation coefficients (r_p) were used to determine the relationship between UTI values, urinary protein content and the concentration of α_1PI-elastase complexes, respectively.

Student's t-test was taken to characterize the differences in UTI values between the different lung dis-

eases. When p-values were less than 0.05, differences were considered to be significant.

RESULTS

Preparation of monoclonal Antibodies

Monoclonal antibodies (mAbs) were derived from fusions of mice immunized with the high molecular weight ITI molecule. They were recognized by screening in ELISA assays for murine IgG antibodies. The antibodies obtained were tested for their reactivity with the intact ITI molecule as well as low molecular weight inhibitory active fragments. Two antibodies (IATI4 and IATI12) were found to react with high molecular weight ITI, but not with low molecular weight inhibitory active fragments. It was therefore concluded, that these antibodies recognize epitopes located out of the inhibitory active domains of ITI (21). Another mAb, IATI5, which is decribed in this paper, was found to react with both, ITI and its inhibitory active fragments. Its specificity was confirmed by immunoblotting analysis using ITI- and HI30-preparations as well as urine samples from patients suffering from lung diseases.

Immunoblotting Analysis

In order to characterize the ITI forms recognized by the monoclonal antibody IATI5, serum-ITI, purified HI-

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30, and urine of a patient suffering from lung empyema were analysed by SDS-PAGE followed by either protein staining or by immuno-blotting using the antibody. The reaction of IATI5 with ITI is shown in Fig.1, lane B. IATI5 reacts with three distinct proteins with apparent molecular masses of 240 kDa, 120 kDa, and 50 kDa, respectively. In lanes E and G the reaction pattern of IATI5 with purified HI-30 and urine is depicted. In the HI-30 preparation mAb IATI5 reacts with a 33 kDa protein (lane E). MAb IATI5 reacts also with a minor band of lower molecular weight which might represent a lower molecular weight fragment of HI-30 contained within the preparation (lane E). This band was not visible by protein-staining (lane D) due to its low concentration. In concentrated urine mAb IATI5 reacted with a compound of 33 kDa (Fig. 1, lane G) comigrating with a faint protein band of the same molecular weight (Fig. 1, lane F, arrow) and with the major immuno-stained band in the HI-30 preparation (Fig. 1, lane E). Whether the very faintly staining bands of ≈ 35 kDa (above HI-30) are due to nonspecific staining or not can not be decided at the moment.

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Enzyme-linked Immuno-sorbent Assay for UTI in Urine

Purified monoclonal antibody IATI5 and a polyclonal anti-HI-30 immunoglobulin preparation raised in rabbits

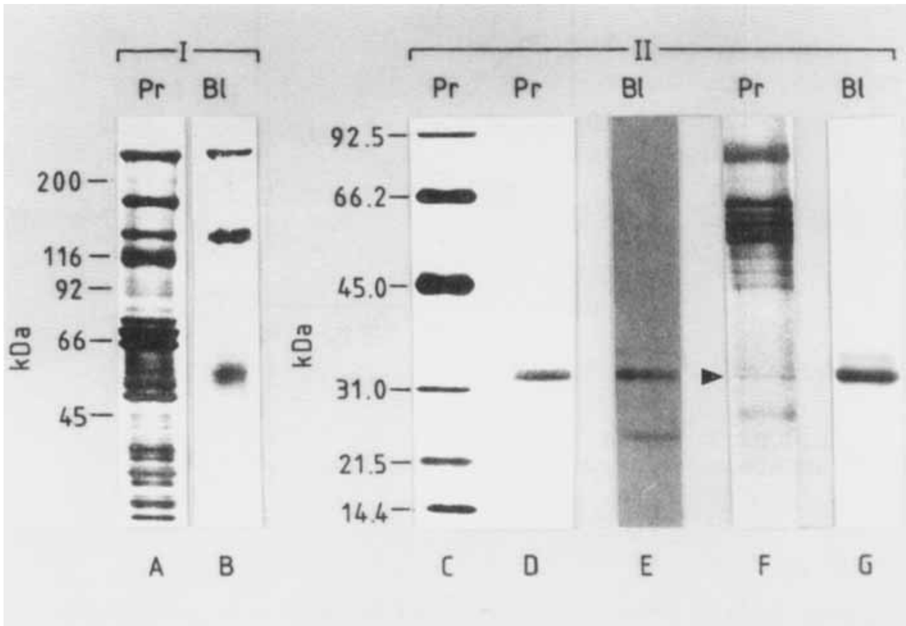

FIGURE 1: Protein pattern (Pr) obtained by silver staining as well as corresponding proteins recognized by anti-HI-30 mAB clone IATI5 after immunoblotting (Bl).

A,B: plasma-derived ITI-preparation
C : molecular weight marker proteins
D,E: purified HI-30
F,G: urine from a patient with lung empyema

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were used to establish a solid phase enzyme immuno assay for UTI. The assay system is detailed above under "Materials and Methods". Standardization experiments revealed a linear relationship between the concentration of HI-30 and the optical density in the concentration range between 10 and 0.5 ng/ml HI-30 (Fig.2).

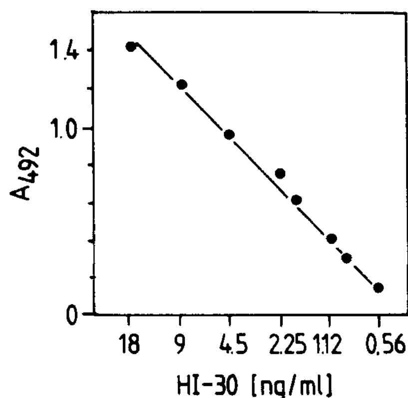

FIGURE 2: Standard curve of UTI-ELISA obtained by analysis of graded amounts of purified HI-30.j

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Linear slopes were also obtained by analysis of UTI in urine samples pretreated with perchloric acid, indicating that in fact acid-soluble compounds were quantified. The curves obtained with urine samples were parallel to the standard curve.

The assay system was taken to analyse urine samples of either a group of healthy individuals without evidence for pulmonary disease or of patients suffering from lung empyema, lung tuberculosis or severe malignant lung disease. Creatinine was determined in each urine sample in order to standardize differences in urine dilution. The UTI values detected by ELISA were thus given as µg UTI/mg creatinine. Using perchloric-acid-treated urine samples, an intraassay coefficient of

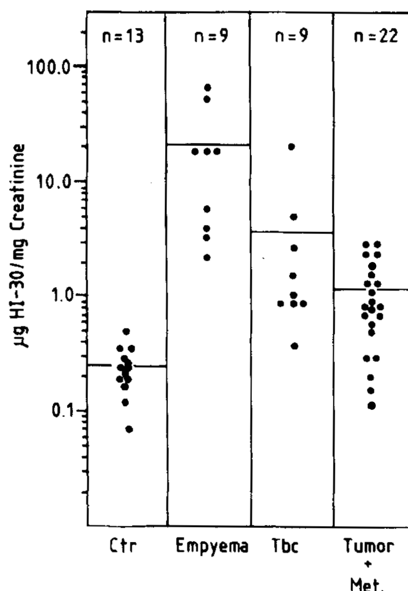

FIGURE 3: Detection of UTI in urine of healthy individuals and patients suffering from severe inflammatory and malignant lung diseases.

Ctr : Control group of healthy individuals
Empyema : Patients with lung empyema
Tbc : Patients with lung tuberculosis
Tumor + Met.: Patients with primary and secondary lung carcinoma

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variation of 6% was calculated. The interassay coefficient was found to be 12%.

As shown in Fig. 3 increased levels of UTI were observed in patients with inflammatory and malignant lung disease as compared to the control group (Ctr). UTI values of all types of lung diseases investigated were significantly higher as compared to the control group

(p = 0.0006 for lung tumor patients, p = 0.0318 for lung tuberculosis and p = 0.0020 for empyema, respectively). Highest UTI values were observed in the urine of patients suffering from lung empyema.

Besides UTI, urinary protein contents as well as the levels of α_1PI-elastase complexes in plasma were determined in the same groups of patients. Protein levels were found to be elevated in all groups of patients suffering from lung diseases as compared to the control group. However, the differences in the urinary protein contents between the groups of several lung diseases were not significant.

α_1PI-elastase complex values in plasma of patients suffering from various lung diseases were also elevated as compared to the group of healthy subjects.

The mean values and standard deviations of UTI-, urinary protein- and α_1PI-elastase complex-levels are shown in table 1. Further statistical analysis revealed that UTI levels were not correlated with (i) total protein content in urine and (ii) the plasma levels of α1PI-elastase complexes.

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DISCUSSION

In this report we present our data on the developement of a ELISA for the immunological quantification of UTI (urinary HI-30), an ITI related peptide. The assay is

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TABLE 1

Mean Values and Standard Deviations of UTI Level, urinary Protein Content, and Level of α_1PI-elastase Complex in Plasma of Patients with severe Lung Diseases in Comparison to a Control Group of healthy Individuals

Group	(n)	UTI [μg/mg creatinine]	urinary protein content [μg/mg creatinine]	α_1PI-elastase complex [μg/ml]
control	(13)	0.25 ± 0.11	62.62 ± 24.56	34.7 ± 2.2
empyema	(9)	21.45 ± 23.95	316.63 ± 221.53	59.6 ± 15.0
tuberculosis	(9)	3.76 ± 6.52	184.46 ± 154.94	59.9 ± 19.5
tumor	(22)	1.14 ± 0.89	138.17 ± 117.07	140.0 ± 112.9

based on a monoclonal anti-HI-30 antibody produced by clone IATI5 recently raised in our laboratory. Quantification of perchloric acid soluble UTI in urine of patients with inflammatory and malignant lung diseases was chosen to describe the characteristica of the assay. In contrast to functional and biochemical methods the described assay system allows efficient testing of a great number of test samples under routine conditions. The intra-assay-cofficient and the interassy coefficient were found to be in an acceptable range. The test was not distored by the presence of nonspecific proteins, because parallel curves were obtained with the purified HI-30 standard and the perchloric acid treated urine.

MAb IATI5 recognizes three major bands in a conventionally purified ITI preparation which contains - besides 240 kDa intact ITI - several ITI related peptides, as revealed by protein staining (Fig. 1, lane A). This was not unexpected since several authors found that ITI in purified form is unstable and tends to disintegrate into peptides of lower molecular weight (reviewed in 3). Recently Gebhard et al. (7) reported that ITI is composed of three different protein components with molecular weights of 56 kDa, 78 kDa and 85 kDa, respectively. The protein component of 56 kDa was found to contain the inhibitory activity against

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proteinases whereas the other two components were not inhibitory. A 130 - 140 kDa protein found in ITI preparations was interpreted to be a complex of the 56 + 78 kDa or the 56 + 85 kDa compounds whereas the native 240 kDa ITI complex is thought to be a complex of all three components with as yet unknown (possibly variable) stoichiometric composition (7).

In line with these explanations our immuno-blot data demonstrate that mAb IATI5 recognizes an epitope located at the 56 kDa inhibitory compound. As expected it detects three distinct bands in the ITI preparation (Fig. 1 lane B): (i) a 240 kDa immuno-reactive protein band representing the native ITI complex comprising the 56 kDa compound, (ii) a 120 kDa immuno-reactive protein band representing a complex between the 56 kDa component and either the 78 kDa or the 85 kDa compound, and (iii) the lowest molecular weight immuno-reactive peptide (about 50 kDa) which is the single, i.e. non-complexed, inhibitory compound.

In the purified HI-30 preparation derived from ITI after trypsin treatment (Fig. 1, lane D) and in urine (Fig. 1, lane G) mAb IATI5 detected a protein of about 33 kDa (arrows). The molecular weight difference to the approx. 50 kDa component observed in native ITI (Fig. 1, lane B) could be due to either degradation resulting from trypsin treatment during preparation of HI-30 used

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as a standard (in urine, urinary proteases, such as uropepsin , could be reponsible for proteolytic processing of UTI (3)) or to a difference in the degree of glycosylation. No high molecular protein-bands were found to give a positive reaction with IATI5 in western blot experiments. This indicates, that no complexes between UTI and proteinases are present in the urine samples.

Quantification of UTI by our new ELISA indicated that urinary excretion of HI-30 is massively increased during inflammatory lung disease, i.e. empyema of the lung in particular (see Fig. 2). A more moderate increase was observed in patients suffering from lung tuberculosis or from severe malignant lung disease. Quantification of total protein in urine revealed elevated levels in the groups of patients with lung diseases, but protein levels were < 300 µg/ml excluding renal dysfunctions which may also result in increased UTI excretion. Moreover, no correlation was observed when the protein concentrations were correlated to UTI levels. This means that the increaseed UTI-concentrations in urine might reflect an increased release of this component from the ITI-complex as a result of a higher poteolytic burden accompained with the inflammatory and malignant diseases. In this context Pratt et al. (22) could show in a recently published

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study, that the neutrophilic enzymes elastase and cathepsin G as well as intact neutrophils are able to liberate HI-30 from intact ITI in vitro.

In view of this findings we analysed whether HI-30 levels in urine were correlated with the level of α_1PI-elastase complexes in patient plasma. Although the α_1PI-elastase complexe levels were like UTI levels elevated in samples of patients with different lung diseases, no correlation was observed between the level of α_1PI-elastase complexes in plasma and UTI-levels. There are two possible explanations: (i) other - as yet undefined - proteolytic enzymes, could be responsible for the release of HI-30. Especially in the group of patients suffering from empyema, which show the highest UTI values, procaryotic proteases of the causative agents could be able to release HI-30 fom ITI; in the case of patients suffering from primary and secondary lung tumors tumor-associated proteases besides those released during inflammatory processes could be responsible for HI-30 release. (ii) the increase of ITI may be a result of an acute-phase-like response involving the biosynthesis of the inhibitory activity containing component of ITI rather than decomposition of high molecular weight ITI (9).

Counteraction of leukocytic enzymes was thought to be the function of HI-30. However, the determination of

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inhibition equilibrium constants (22-24) indicated that HI-30 is a rather weak inhibitor for neutrophilic elastase and cathepsin G. In conclusion HI-30 might well exert other functions than controlling leukocytic enzymes. Most interestingly in this context it has been reported by McKeehan et al. (25) that HI-30 can function as a endothelial cell growth factor. Thus, HI-30 could function as a angiogenetic factor during severe inflammation or tumor formation.

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Although the mechanism of the formation of UTI as well as its function are unclear up to now, we think that our ELISA will be a useful tool to answer questions regarding regulation and release of HI-30 into urine during several diseases in future.

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