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Endotoxaemia in Patients with *Crohn's* Disease: A Longitudinal Study of Elastase/ α_1 -Proteinase Inhibitor and *Limulus*-Amoebocyte-Lysate Reactivity

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Summary: During intensive care, the severity of gut-derived systemic endotoxaemia in 16 patients with active *Crohn's* disease was characterized by the simultaneous determination of elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity. Using both assays in 15/16 patients the detectable endotoxic activity during the disease course was reflected by a parallelism between elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity. In 8 gut-irrigated patients, significantly lower elastase/ α_1 -proteinase-inhibitor concentrations and *Limulus*-amoebocyte-lysate-reactivities were measured compared with 8 gut-nonirrigated patients. The *Crohn's* disease activity index and the *van Hees* activity index as well as the inpatient time were significantly lower in lavage patients compared with gut-nonirrigated patients. It is concluded with that elastase/ α_1 -proteinase-inhibitor can be used as an alternative to the bioassayed *Limulus*-amoebocyte-lysate-reactivity in order to characterize systemic endotoxaemia.

Introduction

In patients with *Crohn's* disease, the *Gram*-negative enterobacteriaceae (*Escherichia coli*, *Salmonella*, *Proteus*, *Klebsiella*, *Enterobacter*, *Serratia*, *Yersinia*) as well as their cell wall constituents, the lipopolysaccharides (endotoxin), contribute to the inflammatory lesions in the bowel wall (1). These are mainly caused by abnormal and invasive bacterial colonisation (2–6). During the acute phase of *Crohn's* disease, a systemic endotoxaemia (7–10) and elevated antibody concentrations directed against *Escherichia coli*-O serotypes (11, 12), and lipid A (13–15), the toxophor within the lipopolysaccharide molecule (16), were found.

Endotoxin induces the release of elastase and other inflammation triggering substances (cathepsin, collagenase, myeloperoxidase) from mononuclear phago-

cytes (17, 18). The proteolytic inflammatory effect of elastase is generated when the amount of α_1 -proteinase-inhibitor does not suffice to neutralize the elastase activity (19).

Lipopolysaccharides also induce a leukocytosis in patients with *Crohn's* disease (20, 21) concomitantly with a neutrophilia and a lymphocytopenia (22, 23). In the damaged bowel wall a massive infiltration of granulocytes, macrophages, cytotoxic-T-cells, B- and plasma-cells was shown (24–26).

Based upon these inter-related features it was of interest to determine elastase/ α_1 -proteinase-inhibitor in plasma of lipopolysaccharide-stimulated whole blood from healthy adults and patients with *Crohn's* disease. The extent of endotoxaemia in lavage and gut-nonirrigated patients (27) with active *Crohn's* disease was characterized by the simultaneous application of the

turbidimetric *Limulus*-amoebocyte-lysate-reactivity test (28, 29) and the elastase/ α_1 -proteinase-inhibitor enzyme-linked-immunosorbent assay (30, 31) as well as by the *Crohn's* disease activity index (32) and the *van Hees* activity index (33).

Materials and Methods

Specimens

From 16 patients (male/female ratio: 7/9, age range: 17–48 years), with active *Crohn's* disease confirmed by clinical, radiological, endoscopic and pathohistological features, the course of endotoxaemia was assayed during 14 days of inpatient time. All patients were administered total parenteral nutrition, 9620 kJ/24 h in a volume of 2500 ml through a central venous catheter at the day of admission for at least one week. As a standard therapeutic regimen prednisone and sulphasalazine were given orally to all patients.

For 8 lavage patients, 2 whole gut-irrigations were performed by placing a radiopaque polyvinyl tube into the jejunum (34). NaCl solution (0.15 mol/l) containing 5-acetyl-salicylic acid was used as lavage fluid at 37 °C in the first week (27).

Serum sodium, potassium, bicarbonate, haemoglobin, haematocrit and body weight were determined before, during and after whole gut-irrigation. For randomisation, cards were prepared, which indicated to which treatment group the patients were allocated. 8/16 patients allocated to the lavage group gave informed consent before treatment.

From a 59 year-old intensive care patient samples were drawn in 2 h intervals to study the 24 h-profile between elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity.

Fifty clinically apparent healthy adults (male/female ratio: 23/27, age range: 18–64 years), who were routinely examined by the personnel medical service, served to set up a reference range for elastase/ α_1 -proteinase-inhibitor. Laboratory data for these individuals based on haemogram (erythrocyte and leukocyte count, haemoglobin, haematocrit), aspartate aminotransferase, alanine aminotransferase, serum creatinine, urea, hepatitis B surface antigen, anti-hepatitis B surface antibody showed normal values in all cases.

From all patients and healthy adults heparinized (50 USP, Heparin-Novo, lot. No. B 551*13, Novo-Industries D-6500 Mainz) venous blood was obtained by sterile venepuncture (29). After centrifugation of whole blood at 150 g for 10 min at 20 °C the plasma supernatants were pipetted into sterile LAS-R-borosilicate glass cuvettes 10 × 75 mm (cat. No. RD 086-172, Hyland Travenol, D-8000 Munich) and stored at –30 °C until use.

To test the influence of various anticoagulants on the elastase/ α_1 -proteinase-inhibitor assay, whole blood from 10 healthy adults was anticoagulated either with heparin, or K₂-EDTA, 0.24 mol/l (cat. No. 03258) or citrate, 0.1 mol/l (cat. No. 05.268, Sarstedt, D-5223 Nümbrecht). After different storage times (10 min, 30 min, 60 min) and incubation temperatures (4 °C, 20 °C, 37 °C) the elastase/ α_1 -proteinase inhibitor content in plasma was assayed.

To test the release of elastase into plasma from unstimulated and lipopolysaccharide-stimulated whole blood, additional samples from 10 healthy adults and 5 patients with untreated active *Crohn's* disease were obtained. After incubation with 20 µg of lipopolysaccharide (lipopolysaccharide from *Escherichia coli* 055:B5, lot. No. 3120 Difco-Lab., Detroit, USA) for 30 min at 37 °C elastase/ α_1 -proteinase-inhibitor was determined in plasma.

Elastase/ α_1 -proteinase-inhibitor assay

The elastase/ α_1 -proteinase-inhibitor assay (lot. No. 41292105), standards (1 µg/l, 2 µg/l, 4.5 µg/l, 11.9 µg/l) as well as the human control plasmas (106 µg/l, 153 µg/l) were kindly supplied by Dr. S. Neumann (Biochemische Forschung Merck, D-6100 Darmstadt). The working procedure of the elastase/ α_1 -proteinase-inhibitor assay was performed unmodified as described elsewhere (30, 31).

Limulus-amoebocyte-lysate-test

Lyophilized *Limulus*-amoebocyte-lysate-reagent (lot. No. 52-76-245, Associates of Cape Cod, Woods Hole, MA, USA) and lipopolysaccharide-standard (Novo-Pyrexal forte, 1.0 mg/l, Pyroquant Diagnostik, D-6082 Walldorf) (35) were used for all samples. The turbidimetric assay procedure was performed as described elsewhere (28, 29). The absorbance values at 365 nm expressing the amount of lipopolysaccharide activity in the sample were plotted against the absorbance values of the lipopolysaccharide calibration curve thus expressing the *Limulus*-amoebocyte-lysate-reactivity in the sample as lipopolysaccharide-equivalents.

Statistical analysis

For evaluation of results imprecision within-run and between-days, *Student's* t-test ($p < 0.025$), multi-variate variation analysis ($p < 0.05$), coefficient of correlation (r) and *Spearman's* coefficient of rank correlation (r_s) were performed as described elsewhere (36).

Results

Effect of various anticoagulants, storage times and temperatures on elastase/ α_1 -proteinase-inhibitor

No significantly different ($p > 0.05$) elastase/ α_1 -proteinase-inhibitor concentrations were found in plasma from heparinized, K₂-EDTA- or citrate-containing whole blood samples, stored for 10, 30 or 60 min at 4 °C or 20 °C (tab. 1). In contrast, a significantly ($p < 0.01$) elevated elastase/ α_1 -proteinase inhibitor concentration was determined in plasma from whole blood stored 60 min compared with 10 min or 30 min, at 37 °C.

Elastase/ α_1 -proteinase-inhibitor in plasma of lipopolysaccharide-stimulated whole blood

Increased plasma elastase/ α_1 -proteinase-inhibitor ($p < 0.0005$) was found in lipopolysaccharide-stimulated whole blood (patients with *Crohn's* disease: 1154.6 ± 157.3 µg/l; healthy adults: 231.5 ± 74.6 µg/l) compared with unstimulated whole blood (patients with *Crohn's* disease: 478.0 ± 162.5 µg/l; healthy adults: 92.1 ± 14.0 µg/l).

Table 1. Plasma-elastase/ α_1 -proteinase-inhibitor in whole blood samples (n = 10) from healthy adults containing various anti-coagulants and treated with different storage times and incubation temperatures ($\bar{x}$ = mean value, s = 1 standard deviation).

Temperature conditions	Storage times Anti-coagulants	10 min			30 min			60 min		
		Heparin	K ₂ -EDTA	Citrate	Heparin	K ₂ -EDTA	Citrate	Heparin	K ₂ -EDTA	Citrate
4 °C	$\bar{x}$	78.3	78.4	78.6	78.5	77.9	78.4	80.8	80.3	80.1
	s	27.5	27.0	26.7	25.9	26.3	26.0	26.1	26.8	27.6
20 °C	$\bar{x}$	81.8	82.2	82.5	82.8	84.1	83.8	84.6	84.9	84.1
	s	25.1	26.8	26.9	25.8	27.6	26.0	26.2	25.1	27.4
37 °C	$\bar{x}$	85.0	85.1	84.5	85.7	85.5	84.2	124.8	128.9	131.0
	s	25.4	25.7	26.5	25.6	24.2	25.4	30.8	26.0	30.4

Characterization of endotoxaemia by elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity

Prior to the longitudinal study in patients with *Crohn's* disease, a 24 h-profile of elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity was provided for one septic intensive care patient, who had a positive (*Escherichia coli*) blood culture and leukocyte counts in the range of 7.2 – 8.7 $\times 10^9/l$. A parallel course ($r = 0.79$) between elastase/ α_1 -proteinase-inhibitor and positive *Limulus*-amoebocyte-lysate reactivities was found.

The same parallelism ($0.68 > r < 0.99$; $0.60 > r_s < 1.00$) between elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity was found for 15/16 lavage- and gut-nonirrigated patients with *Crohn's* disease. Two representative courses are depicted in figure 1 and 2.

Lavage patients revealed significantly lower ($p < 0.025$) mean values of elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity at day 6 and 8 than gut-nonirrigated patients (figs. 3, 4). In accordance with the course of elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity, the *Crohn's* disease activity index and *van Hees* index decreased more rapidly ($p < 0.025$) in the lavage group (*Crohn's* disease activity index: 228 ± 39 index points to 86 ± 42 index points; *van Hees*: 214 ± 33 index points to 157 ± 33 index points) than in gut-nonirrigated patients (*Crohn's* disease activity index: 240 ± 46 index points to 158 ± 53 index points; *van Hees*: 233 ± 46 index points to 191 ± 28 index points). Patients from the lavage group (25.2 ± 8.5 days) were discharged earlier from the hospital than gut-nonirrigated patients (39.1 ± 11.9 days) ($p < 0.01$).

Discussion

In patients with active inflammatory bowel disease and intestinal mucosal lesions the enteral resorption of lipopolysaccharides results in portal endotoxaemia (37), due to insufficient clearance of lipopolysaccharides by the hepatic mononuclear phagocytic system (38, 39). Endotoxin may also reach the blood stream by transmural escape through the peritoneal cavity or propagation via intestinal lymphatics and the ductus thoracicus (8).

A systemic endotoxaemia based on single determinations of positive *Limulus*-amoebocyte-lysate-reactivity was found in all patients with active *Crohn's* disease (8, 9). In contrast, *Kruis* et al. (14) detected a positive *Limulus*-amoebocyte-lysate-reactivity only in a minority of patients. The cause of the above mentioned discrepancy may be the interference of *Limulus*-amoebocyte-lysate-reactivity, with polynucleotides, peptidoglycans derived from cell walls of Gram-positive bacteria, plasma macromolecules (HDL, phospholipides, inhibitors) and leukocytic factor(s) (28, 29) as well as *Limulus*-amoebocyte-lysate-batch variabilities. Thus, single determinations of *Limulus*-amoebocyte-lysate-reactivity are of limited clinical validity in characterizing "endotoxaemia" (29). Therefore, replicate determinations of the *Limulus*-amoebocyte-lysate-reactivity were performed in order to monitor the patient-specific course of endotoxaemia.

Lipopolysaccharides also effect the release of elastase from leukocytes (18, 19). Free elastase amplifies the inflammatory potency of lipopolysaccharides (21, 40) by cleavage of clotting-, complement- and fibrinolytic-factors, immunoglobulins, transport proteins and plasma protein inhibitors (41–47) as well as by destruction of collagen, elastin and proteoglycans (48).

Fig. 1. Elastase/ α_1 -proteinase-inhibitor, *Limulus*-amoebocyte-lysate-reactivity and clinical data in a gut-irrigated patient with active Crohn's disease during the inpatient time (28 days).

Patient S. B. (22.5 years, female), 7–8 diarrhoeas/day, 4 kg body weight loss, chill and fever before hospital admission.

Pathohistological area of inflammation: terminal ileum, colon.

		At hospital admission	At discharge
Body weight	(kg)	52	61
Fever	(°C)	39	37
Erythrocyte sedimentation rate	(mm)	22/48	25/56
Crohn's disease activity index	(points)	231	118
van Hees activity index	(points)	282	150
Leukocyte count	($10^9/l$)	3.6	5.6
Cholinesterase	(U/l)	580	2050
Total protein	(g/l)	52	65
Protein fractions			
albumin		0.49	0.57
α_1 -globulin		0.06	0.05
α_2 -globulin		0.13	0.11
β -globulin		0.10	0.15
γ -globulin		0.22	0.12

Treatment: conservative therapy and 2 whole gut-irrigations at days 3 and 5.

Elastase/ α_1 -proteinase-inhibitor ■ — □
Limulus-amoebocyte-lysate-reactivity ● — ●

Based upon this pathochemical interrelationship, *Limulus*-amoebocyte-lysate-reactivity and elastase/ α_1 -proteinase-inhibitor were determined in parallel to characterize the course of endotoxaemia.

The precision data ($n = 10$) for elastase/ α_1 -proteinase-inhibitor (coefficients of variation: imprecision within-run 3.6%; imprecision between-days 9.0%) and the

Fig. 2. Elastase/ α_1 -proteinase-inhibitor, *Limulus*-amoebocyte-lysate-reactivity and clinical data in a gut-nonirrigated patient with active Crohn's disease during the inpatient time (38 days).

Patient S. H. (23.5 years, male), 10–12 diarrhoeas/day, 8 kg body weight loss before hospital admission.

Pathohistological area of inflammation: terminal ileum, colon.

		At hospital admission	At discharge
Body weight	(kg)	62	72
Fever	(°C)	37	37
Erythrocyte sedimentation rate	(mm)	55/80	10/20
Crohn's disease activity index	(points)	302	156
van Hees activity index	(points)	227	113
Leukocytes	($10^9/l$)	15.9	8.9
Cholinesterase	(U/l)	1200	2150
Total protein	(g/l)	51	66
Protein fractions			
albumin		0.50	0.70
α_1 -globulin		0.07	0.04
α_2 -globulin		0.12	0.07
β -globulin		0.11	0.10
γ -globulin		0.20	0.10

Treatment: conservative therapy.

Elastase/ α_1 -proteinase-inhibitor ■ — □
Limulus-amoebocyte-lysate-reactivity ● — ●

obtained reference range (95% percentile: $< 106 \mu g/l$) were in accordance with other authors (30, 31, 49). The practicability (e.g. influence of anticoagulants, storage- and temperature conditions) of the elastase/ α_1 -proteinase-inhibitor assay showed that in heparin- K_2 -EDTA- or citrate-containing plasma, similar elastase/ α_1 -proteinase-inhibitor concentrations were detectable. These findings were the prerequisite for using

Fig. 3. Elastase/ α_1 -proteinase-inhibitor concentrations in patients with Crohn's disease (mean values). Lavage patients $\circ$ — $\circ$, gut-nonirrigated patients $\blacksquare$ — $\blacksquare$

a single specimen for both the elastase/ α_1 -proteinase-inhibitor-assay and the turbidimetric *Limulus*-amoebocyte-lysate-reactivity assay. In agreement with *Duswald* (50), plasma elastase/ α_1 -proteinase-inhibitor increased by less than 10% up to 60 min at 4 °C or 20 °C. A significantly elevated plasma elastase/ α_1 -proteinase-inhibitor was observed only after a 60 min interval at 37 °C between blood sampling and centrifugation. This assay was performed to differentiate between the 37 °C-mediated elastase release and the lipopolysaccharide-mediated release of elastase. In the in vitro studies with a 30 min incubation time, the found elastase/ α_1 -proteinase-inhibitor-concentrations were primarily caused by lipopolysaccharide-stimulated leukocytes. Although there was a difference between elastase values of unstimulated and lipopolysaccharide-stimulated whole blood from healthy adults and patients with Crohn's disease the relative elastase increase was similar for both groups.

Similar results were reported by *Duswald* (50) and *Aasen et al.* (51) for healthy adults and dogs respectively.

To confirm the in vitro findings, elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity were determined in a septic intensive care patient with an *Escherichia coli* bacteraemia. The observed parallel profile between elastase/ α_1 -proteinase-inhibitor and *Limulus*-amoebocyte-lysate-reactivity for this patient and the patient with active Crohn's disease suggest that although different analytes were determined, both tests measured the underlying endotoxic activity simultaneously in a similar manner. Elastase/ α_1 -proteinase-inhibitor seemed to be a more adequate marker for the inflammatory disease activity than the leukogram, since in the bacteraemic septic patient

Fig. 4. *Limulus*-amoebocyte-lysate-reactivities in patients with Crohn's disease (mean values). Lavage patients $\circ$ — $\circ$, gut-nonirrigated patients $\blacksquare$ — $\blacksquare$

with leukocyte values within the reference range, elastase/ α_1 -proteinase-inhibitor levels were found to be constantly elevated.

The constantly lower elastase/ α_1 -proteinase-inhibitor concentrations and *Limulus*-amoebocyte-lysate-reactivities as well as the more rapid reduction of endotoxic activity in lavage patients, compared with gut-nonirrigated patients, support the hypothesis that the reduced endotoxaemia in lavage patients was due to a reduction of intestinal lipopolysaccharide biomass. In agreement with our findings a reduction of intestinal bacterial flora was also reported by *Hewitt et al.* (52) after lavage treatment. The difference of endotoxic activity in lavage- and gut-nonirrigated patients was not the result of a dilution effect by the orthograde saline gut-irrigation, since serum sodium, potassium, bicarbonate, haematocrit, haemoglobin and body weight were stable before, during and after whole gut-irrigation.

In agreement with our results, *Adeyemi et al.* (53) found, in patients with active Crohn's disease, elevated elastase/ α_1 -proteinase-inhibitor compared with healthy adults and patients with inactive Crohn's disease. Their results yielded a positive correlation between the elastase/ α_1 -proteinase-inhibitor and the Crohn's disease activity index. In conclusion the longitudinal study showed that

- elastase/ α_1 -proteinase-inhibitor may be used as an alternative to the *Limulus*-amoebocyte-lysate-reactivity and
- both tests as well as the Crohn's disease activity index and *van Hees* activity index yielded a lower degree of endotoxaemia and a reduced inpatient time in lavage patients, compared with gut-nonirrigated patients.

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