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Questioning Current Concepts in Acute Pancreatitis: Endotoxin Contamination of Porcine Pancreatic Elastase Is Responsible for Experimental Pancreatitis-Associated Distant Organ Failure

Fabian Geisler, Hansa Aligi, Marc Riemann, and Roland M. Schmid

The systemic inflammatory response syndrome is responsible for pancreatitis-associated mortality. Recent in vitro and in vivo studies have suggested that pancreatic elastase is one missing link between the localized inflammatory process in the pancreas and distant organ dysfunction and failure. It has been shown that pancreatic elastase activates transcription factors, including NF-κB, and induces TNF-α secretion in myeloid cells via TLRs. In this study we demonstrate that a highly purified low endotoxin pancreatic elastase preparation (El-UP) failed both to activate NF-κB and to induce TNF-α release in RAW 264.7 cells and bone marrow-derived macrophages. In contrast, a less purified elastase preparation (El-IV) caused activation of NF-κB and was able to induce TNF-α release at very low concentrations. These effects were sensitive to pretreatment of the cells with polymyxin B and were resistant to heat inactivation. Endotoxin activity as determined by the Limulus amebocyte lysate assay was >3 orders of magnitude lower in the low endotoxin elastase preparation (El-UP) compared with less purified elastase preparations (El-IV). In contrast to contaminated elastase or LPS, elastase free of contamination (El-UP) failed to induce elevated serum TNF-α levels or pulmonary neutrophil infiltration after i.p. application in mice and did not induce lethality when coinjected with D-galactosamine. Failure of low endotoxin elastase (El-UP) to induce proinflammatory effects in vivo and in vitro was not due to functional inactivity of the elastase preparation, as determined by elastase activity assay. These results question current concepts of direct proinflammatory effects attributed to pancreatic elastase.

activate TLR4, thus triggering an endogenous pathway to initiate a systemic inflammatory response (17, 18).

TLRs are type I transmembrane proteins that play an important part in innate immunity. TLRs recognize pathogen-associated molecular patterns, such as microbial components, that initiate a range of host mechanisms. In addition, a number of putative endogenous ligands, such as heat shock proteins (Hsp) and oligosaccharides, from the breakdown of extracellular matrix (ECM) have been suggested to be potent activators of the innate immune system (17–24). They all appear to signal through the TLRs, similar to LPS (via TLR4) or bacterial lipoproteins (via TLR2). Recent evidence suggests that pancreatic elastase-induced proinflammatory effects are also mediated by TLR4 (9, 17).

In the present study we show that endotoxin-free pancreatic elastase is not able to stimulate murine macrophages to produce TNF-α, or elicit pulmonary organ injury as observed in acute pancreatitis. Moreover, we demonstrate that the proinflammatory qualities attributed to pancreatic elastase in the current literature might be due to contaminating endotoxin present in the elastase preparations used. Our findings not only have a major impact on acute pancreatitis research, but also affect other inflammatory models (i.e., animal emphysema models) in which pancreatic elastase is used.

Materials and Methods

Materials

Salmonella minnesota R595 derived ultra pure LPS (product 434) was purchased from List Biological Laboratories. Polyoxymyxin B sulfate (product P4932), lyophilized porcine pancreatic elastase type IV (El-I; product E0258), cell culture-tested (not endotoxin-tested) pancreatic elastase (product E7885, prepared from E0258), aqueous suspension of pancreatic elastase type I (product E1250), and β-galactosidase (D-Gal; product G6139) were all from Sigma-Aldrich. High purity low endotoxin (<0.78 endotoxin units (EU)/mg protein according to the manufacturer; 1 mg of protein contains 11.5 U of elastase enzymatic activity) porcine pancreatic elastase (product LE425) was purchased from Elastin Products. For the purpose of this report, high purity low endotoxin elastase from Elastin Products Company is designated El-UP, whereas El-I from Sigma-Aldrich is designated El-I. Culture-tested elastase from Sigma-Aldrich is designated El-I/IV-2, and aqueous suspension of elastase type I is designated El-I. The definition of 1 U for all elastase preparations used is the hydrolysis of 1 μmol/min of the substrate N-succinyl-t- Ala-Ala-p-nitroanilide (N-suc-3Ala-pNa) at 37°C, pH 5.0. To minimize loss of enzymatic activity, all lyophilized elastase preparations were dissolved in 0.1 M NaCl containing 0.05 M NaOAc, pH 5.0, and further diluted in PBS immediately before the start of the experiments.

Preparation of whole-cell protein extracts

For preparation of whole-cell protein extracts, RAW 264.7 cells or BMDM were washed in ice-cold PBS and then removed with a rubber policeman in 300 μl of Nonidet P-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% SDS, 1% Triton X-100, and 0.4 mM PMSF). The lysate was then incubated for 10 min at 4°C, cleared by centrifugation (14,000 rpm, 1 min, 4°C), snap-frozen in liquid nitrogen, and stored at −80°C until assayed.

SDS-PAGE and Western blotting

Whole-cell protein extracts were fractionated by discontinuous SDS-PAGE (10% separating gel) and transferred to 0.45-μm pore size polyvinylidene difluoride membranes (Schleicher & Schuell). Equal loading amounts of protein were confirmed by Coomassie Blue staining. The membranes were blocked for 1 h in skim milk 5% (w/v) in PBS and 0.05% Tween 20 (T-PBS) and then incubated for 1 h with Abs against IκBα or IkBβ (C20 and C21; Santa Cruz Biotechnology) at a dilution of 1/1000 in skim milk 5% (w/v) in T-PBS. After washing membranes three times with T-PBS, membranes were incubated for 60 min with a secondary goat anti-rabbit IgG HRP Ab (Dianova-Immunotech) diluted at 1/5000 in 5% (w/v) skim milk T-PBS. Blots were washed three times with T-PBS and developed with ECL reagents (Amersham Biosciences).

EMSA

The DNA-probe used for EMSAs corresponded to the high affinity κB sequences found in the mouse κ-L chain enhancer and in the HIV-1 promoter region. Two oligonucleotides were annealed to generate a double-stranded probe: sense, 5′-AGCTTGGGGGACTTCCACATGAGC-3′; and antisense, 5′-AATCTGCACTAGTGAAAATCCCA-3′ (binding site is underlined). The probe was labeled with [32P]dATP and purified on phosphocellulose columns (Sephadex G-25; Amersham Biosciences). Labeled probe (30,000 cpm) was added to 10 μg of whole-cell protein extract in the presence of 5 μg of poly(di-c). Binding reactions were conducted in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 4% glycerol for 30 min at room temperature. DNA protein complexes were resolved by electrophoresis on a 4% (w/v) polyacrylamide gel. Gels were vacuum-dried and exposed to Kodak BioMax MX-1 film at −70°C with intensifying screens.

Measurements of endotoxin activity

The endotoxin activities of different pancreatic elastase preparations were determined using a Limulus amebocyte lysate (LAL) assay kit (QCL-1000, catalogue no. 50–647U; BioWhittaker) according to the manufacturer’s instructions. Two different charges of elastase preparations were tested.

For generation of bone marrow-derived macrophages (BMDM), C57BL/6N and MyD88-deficient mice were killed by cervical dislocation under ether anesthesia, and bone marrow was collected and cultured as described previously (19). Briefly, bone marrow was flushed from humerus, femur, and tibia and cultured for 6 days in complete RPMI 1640 containing 15% heat-inactivated FCS, 5% heat-inactivated horse serum, and 15% culture supernatant from M-CSF-producing L929 cells.

Cell culture stimulation

For stimulation of RAW 264.7 macrophages, cells were seeded in six-well plates at 8 × 10⁴ cells/well the day before the experiment. BMDM were seeded at 1 × 10⁶ cells/well, respectively. Two hours before stimulation, cells were washed with PBS, and 3 ml of fresh complete culture medium was replaced per well. For cytokine measurements, cells were treated with LPS (1–100 ng/ml) or with different elastase preparations (1–1000 mU/ml).

For TNF-α measurements, media were collected at 2, 4, and 8 h and clarified by centrifugation. Samples were shock-frozen and stored at −80°C. For studying the degradation kinetics of IκBα and IκBβ, cells were treated with LPS (100 ng/ml) or elastase (100 μM) for 10, 15, 30, 60, and 120 min before whole-cell protein extracts for immunoblotting were prepared.

In some experiments, RAW 264.7 cells and BMDM were preincubated with polymyxin B sulfate (50 μg/ml) for 30 min at 37°C before the addition of LPS or different elastase preparations. Likewise, in some experiments, enzyme preparations were boiled at 99°C for 60 min to inactivate elastase activity.

TNF measurements

The TNF-α concentrations in the cell supernatants and serum of mice were determined by a commercially available murine quantitative ELISA kit (Quantikine M; R&D Systems) according to the manufacturer’s instructions. All experiments were conducted with duplicate samples.
Removal of LPS and LPS-associated molecules from pancreatic elastase

Polymyxin B agarose columns (Detoxi-Gel AffinityPak 1-ml columns; Pierce) were used to remove endotoxin from the El-IV preparations according to the manufacturer’s instructions. In brief, columns were washed with 5 column volumes of 1% sodium deoxycholate, followed by 5 column volumes of pyrogen-free water and 5 column volumes of PBS. Elastase (900 μl at 3 U/ml) was applied to each 1-ml Detoxi-Gel column and eluted with PBS in 300-μl fractions after an incubation period of 30 min. Samples were immediately used for cell culture or in vivo experiments, and aliquots were snap-frozen for later determination of endotoxin content.

Animal models

Weight- (16–20 g) and sex-matched mice were injected i.p. with 1.5 U of El-IV or low endotoxin elastase dissolved in 500 μl of PBS, a dose at which systemic inflammatory effects and lung injury have been reported (10, 11, 17). Control animals received PBS or 10 μg of LPS. After 4 h, mice were anesthetized with ketamine, serum was withdrawn from the inferior vena cava, and lung tissue was harvested for histology and determination of neutrophil sequestration as described below. In additional experiments, mice were killed to withdraw blood after 1 h for additional TNF-α measurements. Blood was centrifuged, and the serum was kept at −80°C until assayed.

D-Gal-sensitized animals were monitored hourly for 48 h for lethality after elastase injection. For this, El-IV-1 (1.5 U), low endotoxin elastase (El-UP; 1.5 U), LPS (100 ng), or PBS mixed with 20 mg of D-Gal in a total volume of 500 μl of PBS were injected i.p. In some experiments, El-IV was passed over a polymyxin B column before i.p. application.

Lung myeloperoxidase (MPO) assay

Neutrophil sequestration in lung tissue was quantified by measuring tissue MPO activity. To minimize background MPO activity by remaining non-adherent intravascular blood cells, a needle was inserted into the beating right ventricle to perfuse the pulmonary circulation with PBS until blanching of the lungs occurred. The entire lung was snap-frozen and stored at −80°C until being homogenized on the day of assay in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich) and sonicated three times for 20 s each time. The suspension was subjected to three cycles of freezing and thawing and was centrifuged at 15,000 × g for 10 min, and the resulting supernatant was assayed. The reaction mixture consisted of 200 μl of 10 mM phosphate buffer (pH 6.0), 100 μl of 0.22% guaiacol (Sigma-Aldrich), and 10 μl of the extracted enzyme. The reaction was started with 6 μl of H2O2 (0.1%). The increase in absorbance was monitored spectrophotometrically at 470 nm over 3 min, and the maximum slope of the curve was used to calculate the absorbance. For histological analysis, lungs were fixed via tracheal injection and resuspended with paraformaldehyde. Formalin-fixed tissues were embedded in paraffin. Serial 4-μm-thick sections were stained with H&E and evaluated under light microscopy.

Histologic findings

For histological analysis, lungs were fixed via tracheal injection and resuspended with paraformaldehyde. Formalin-fixed tissues were embedded in paraffin. Serial 4-μm-thick sections were stained with H&E and evaluated under light microscopy.

Statistical analysis

Results were expressed as the mean ± SEM. The level of significance was determined using a two-tailed Student’s t test. Statistical significance was assigned to p < 0.05.

Results

El-IV induces TNF-α secretion and NF-κB activation in macrophages via MyD88

Pancreatic elastase has previously been shown to activate macrophages to produce TNF-α via the nuclear transcription factor NF-κB (8). Moreover, TLRs are reported to be involved in this signaling pathway (9, 17). To test this hypothesis of proinflammatory qualities of pancreatic elastase, TNF-α content was determined in supernatants of mouse wild-type (WT) BMDM and compared with TNF-α content in BMDM from mice deficient for the TLR signaling molecule MyD88 (MyD88−/−). As previously shown, peritoneal macrophages from MyD88−/− mice lack the ability to produce TNF-α, whereas NF-κB activation is delayed upon stimulation with LPS (26). For this approach we used El-IV-1, the elastase preparation used by other authors investigating the proinflammatory actions of pancreatic elastase (11, 17). TNF-α contents in the supernatants were determined after incubation of WT and MyD88−/− BMDM with LPS (100 ng/ml; A) and El-IV-1 (100 μU/ml; B). Values in A and B represent the mean ± SEM of two experiments.

FIGURE 1. Effect of El-IV-1 from Sigma-Aldrich and LPS on TNF-α production in BMDM from WT C57BL/6N and MyD88−/− mice. The TNF-α content of supernatants was determined 0, 2, 4, and 8 h after incubation of WT and MyD88−/− BMDM with LPS (100 ng/ml; A) and El-IV-1 (100 μU/ml; B). Values in A and B represent the mean ± SEM of two experiments.

FIGURE 2. iNOS degradation kinetics in BMDM from WT and MyD88−/− mice in response to LPS and El-IV-1. BMDM were incubated with LPS (100 ng/ml; A) or El-IV-1 (100 μU/ml; B), whole-cell extracts were prepared at the indicated times (minutes), and Western analysis was performed with anti-iNOS Ab.
FIGURE 3. Effect of polymyxin B preincubation on LPS and El-IV-1 responses in RAW 264.7 macrophages. TNF-α content in supernatants was determined 4 h after treatment with LPS (1–100 ng/ml; A) and El-IV-1 (1–100 mU/ml; B) with and without preincubation of the medium with polymyxin B (50 µg/ml). Western analysis was performed for corresponding IκBα degradation kinetics at the indicated times (minutes): LPS (100 ng/ml; C) and El-IV-1 (100 mU/ml; D) without (w/o PB; upper panel) and with (+ PB; lower panel) polymyxin B (50 µg/ml) preincubation. E. The same protein extracts after El-IV-1 (without and with polymyxin B preincubation) were used for EMSA to analyze NF-κB binding activity at the indicated times. Values in A and B represent the mean ± SEM of three experiments.

To evaluate whether TNF-α release from WT and MyD88−/− BMDM in response to LPS or elastase coincides with the activation of NF-κB, whole-cell protein extracts of WT and MyD88−/− BMDM were used to monitor the kinetics of IκB protein degradation. Both LPS at 100 ng/ml and El-IV-1 at 100 mU/ml caused a transient degradation of IκBα within 30 min, followed by a resynthesis of IκBα within 60 min in WT BMDM (Fig. 2, A and B, upper panel). In MyD88−/− BMDM, IκBα degradation was delayed upon stimulation with LPS, showing complete degradation of the inhibitor protein within 60 min and resynthesis within 120 min (Fig. 2A). El-IV-1 also led to delayed kinetics in degrading IκBα in MyD88−/− BMDM (Fig. 2B), suggesting that MyD88 protein and thus TLRs are involved in activating macrophages when exposed to pancreatic elastase. IκB degradation upon incubation with LPS (100 ng/ml) or El-IV-1 (100 mU/ml) both led to degradation of IκBα within 60 min in WT BMDM. In MyD88−/− BMDM, degradation of IκBα was also delayed after incubation with LPS or El-IV-1 compared with WT BMDM (data not shown). Higher concentrations of El-IV-1 (up to 1 U/ml), as routinely used by other research groups, did not alter the kinetics of IκBα or IκBβ degradation (data not shown).

El-IV-1 response in macrophages is inhibited by polymyxin B

The data from WT and MyD88−/− BMDM presented suggest that pancreatic elastase in fact might be an endogenous ligand for TLRs, possibly TLR4. Recently, several putative endogenous ligands of TLRs, such as Hsp60 or Hsp70, had to be re-evaluated for their ability to induce proinflammatory cytokine production by macrophages due to contamination of the substances with LPS or LPS-associated molecules (27–30). Therefore, it had to be ruled out that endotoxin contamination of the pancreatic elastase preparation used was responsible for the observed effects.

To unmask possible endotoxin contamination present in the El-IV-1 preparation, the TNF-α content in supernatants of RAW 264.7 macrophages was determined without and with preincubation of the medium with polymyxin B at 50 µg/ml for 30 min before stimulation with LPS (1–100 ng/ml) or El-IV-1 (1–100 mU/ml) for 4 h. As did BMDM, RAW 264.7 macrophages produced large amounts of TNF-α in response to LPS or El-IV-1 in a dose-dependent manner (Fig. 3, A and B). To our surprise, TNF-α release after stimulation with LPS and that after stimulation with El-IV-1 were markedly inhibited in polymyxin B-pretreated cells (Fig. 3, A and B).

RAW 264.7 macrophages exhibited a similar time course of IκBα degradation as BMDM when exposed to 100 ng/ml LPS or 100 mU/ml El-IV-1 (Fig. 3, C and D, upper panel). Preincubation of the medium with polymyxin B (50 µg/ml) completely inhibited LPS-induced IκBα degradation, whereas degradation of IκBα was delayed and incomplete upon stimulation with El-IV-1 (Fig. 3, C and D, lower panel). The same protein extracts were subjected to EMSA analysis. El-IV-1 caused a time-dependent increase in NF-κB binding activity, with maximum activity after 30 min (Fig. 3E, left panel). NF-κB binding activity was markedly attenuated after preincubation with polymyxin B (Fig. 3E, right panel), suggesting that at least some contaminants might be present in the El-IV-1 preparation.

Heat inactivation of EL-IV-1 does not reduce activation of macrophages

To rule out that unspecific interactions of polymyxin B and El-IV-1 were responsible for the attenuation of the El-IV-1 response

FIGURE 4. Heat inactivation of EL-IV-1 does not reduce activation of RAW 264.7 macrophages. TNF-α production after 4 h in response to El-IV-1 (100 mU/ml) before and after heating the enzyme preparation for 1 h at 99°C without and with preincubation of the media with polymyxin B (50 µg/ml). Values represent the mean ± SEM of three experiments.
in macrophages, pancreatic elastase was inactivated by boiling. In contrast to LPS, pancreatic elastase is a heat-sensitive enzyme. Boiling of the El-IV-1 preparation at 3 U/ml for 60 min caused complete loss of elastase enzymatic activity and degradation of elastase protein, as assessed by elastase enzyme assay (data not shown) and Coomassie Blue-stained SDS-PAGE (data not shown). Nevertheless, this had no significant impact on the TNF-α-inducing activities of the elastase preparation in RAW 264.7 macrophages. Furthermore, the observed TNF-α-inducing effect of the heat-inactivated elastase preparation was still fully sensitive to pre-incubation with polymyxin B (Fig. 4).

All supernatant studies were also performed with different charges of other pancreatic elastase preparations from Sigma-Aldrich, El-IV-2 (cell culture-tested, not endotoxin-tested pancreatic elastase) and El-I (aqueous suspension of pancreatic elastase type I) with both RAW 264.7 macrophages and BMDM with similar results (data not shown). Those two preparations seemed to be even more effective in stimulating macrophages; however, TNF-α release from macrophages was also polymyxin B sensitive and heat resistant.

**Low endotoxin elastase fails to induce TNF-α secretion or activate NF-κB in macrophages**

The polymyxin B sensitivity and failing heat inactivation of the TNF-α-inducing activity of the Sigma-Aldrich elastase preparations suggest that endotoxin contamination might be responsible to a large extent for the observed effects. However, to rule out that possible contaminants mask stimulatory effects caused by pancreatic elastase, cells were also treated with an ultra pure low endotoxin elastase preparation (El-UP) from Elastin Products. As shown in Fig. 5A, the El-IV-1 preparation caused TNF-α release from RAW 264.7 macrophages after 4 h in a dose-dependent manner from 1 to 100 mU/ml. A further increase up to 1000 mU/ml may cause degradation of released TNF-α by elastase. This assumption is supported by our observation that longer incubation periods up to 24 h with high concentrations of elastase (1 U/ml) caused a marked decrease in TNF-α levels in supernatants over time (data not shown). In contrast, the low endotoxin preparation El-UP failed to induce TNF-α release at concentrations up to 1000 mU/ml (Fig. 5A). El-UP (100 mU/ml) also failed to degrade IκBα protein or induce NF-κB binding activity in RAW 264.7 macrophages (Fig. 5B, upper and lower right panels) compared with EL-IV-1 (Fig. 5B, left panel; this figure is identical with EL-IV-1 data from Fig. 3, D and E). EL-UP also did not induce TNF-α secretion (100 mU/ml EL-UP, 2.72 ± 1.8 pg/ml; 1000 mU/ml EL-UP, 4.46 ± 6.3 pg/ml) or degrade IκBα in BMDM (data not shown).

**Low endotoxin elastase has elastolytic activity comparable to other preparations**

To prove that the failure of the low endotoxin preparation to release TNF-α from macrophages was not due to functional inactivity, an elastase activity assay was performed for the low endotoxin preparation (El-UP) and for the two elastase type IV preparations (El-IV-1 and El-IV-2). Fig. 6 demonstrates that all elastase preparations used had similar enzymatic activities on the substrate elastin.

**Low endotoxin elastase fails to induce an inflammatory response in vivo**

Our in vitro experiments suggest that endotoxin contamination might be responsible for stimulating macrophages to produce TNF-α by activating the NF-κB pathway via MyD88. We next addressed the question of whether pancreatic elastase devoid of contamination could still cause inflammatory effects in vivo as described by several authors (10, 11, 17). When injected i.p. into WT mice, LPS (10 μg; n = 3) and El-IV-1 (1.5 U; n = 5) both stimulated a significant increase in serum TNF-α levels after 1 h (Fig. 7A). As expected, TNF-α levels markedly decreased after 4 h (data not shown). In contrast, after EL-UP injection (1.5 U; n = 5), serum TNF-α levels were not significantly different from those in control mice treated with PBS (n = 5; Fig. 7A).

Microscopic examination of lung sections revealed no ultrastructural lesions of the lungs 4 h after i.p. injection of PBS (n =
LPS, El-IV-1, or EL-UP (all \(n/H11005\) 4). However, a marked accumulation of neutrophils predominantly lining the endothelium of the lung microvasculature was observed after LPS and EL-IV-1 treatment (Fig. 7B). This histological picture is very typical for systemic administration of low dose LPS (\(1 mg/kg\)) after 4 h in mice (31, 32). No neutrophils were observed in lungs of EL-UP- or PBS-treated animals (Fig. 7B).

Reflecting histological findings, MPO activity as a measure of pulmonary neutrophil sequestration 4 h after i.p. injection of substances significantly increased after LPS (\(n/H11005\) 4) or El-IV-1 (\(n/H11005\) 5), whereas MPO activity after application of El-UP (\(n/H11005\) 5) was not different from that in PBS-treated control animals (\(n/H11005\) 4; Fig. 7C).

Endotoxin activity present in elastase preparations

To finally prove the suspected contamination of the pancreatic elastase preparations from Sigma-Aldrich, the endotoxin content was quantified by LAL assay. As expected, the endotoxin activity of the low endotoxin elastase preparation was only 0.4 ± 0.1 EU/U (\(n/H11005\) 3). The endotoxin activity of El-IV-1 was 889.5 ± 105.5 EU/U (\(n/H11005\) 4), that of the cell culture-tested elastase preparation (El-I-2) was 1256.8 ± 147.5 EU/U (\(n/H11005\) 4), that of the elastase type 1 solution was 4117.5 ± 1423.4 EU/U (\(n/H11005\) 2), and that of the LPS preparation used was 3014.4 ± 993.7 EU/\(g\) (\(n/H11005\) 5; Fig. 8).

Polymyxin B column: endotoxin removal and protein recovery

Due to the interaction of polymyxin B with the lipid A component of LPS, its use as ligand in affinity sorbents to remove endotoxin from protein solutions has found wide acceptance. However, the clearance factors and protein recovery vary widely depending on the protein solution used (33). We tested whether purification using a polymyxin B column was an appropriate method in terms of endotoxin removal and protein recovery. Fig. 9A shows that passage over the polymyxin B column removed only 90% of the endotoxin activity present in the El-IV-1 preparation (889.5 ± 105.5 EU/U before passage; 93.3 ± 2.8 EU/U after passage), whereas...
and to cause proinflammatory effects by TNF-α release in vivo, as suggested previously (8, 10, 11, 17). We could also show that the TNF-α-inducing activity of three different elastase preparations was entirely due to contaminating endotoxin. Our conclusions are based on the following findings. 1) Highly purified El-UP was unable to degrade IκBα, induce NF-κB binding activity, or stimulate TNF-α release from RAW 264.7 macrophages and BMDM. 2) Contaminated pancreatic elastase preparations (El-IV) caused degradation of IκBα proteins, induction of NF-κB binding activity, and stimulation of RAW 264.7 macrophages to release large amounts of TNF-α comparable to LPS. 3) Preincubation of the medium with polymyxin B attenuated degradation of IκBα and NF-κB binding activity and eliminated the TNF-α-inducing activity of the contaminated elastase (El-IV). 4) The failure of the low endotoxin (El-UP) preparation to induce TNF-α was not due to enzymatic inactivity, because all enzymes used had comparable enzymatic activities. 5) Low endotoxin elastase failed to induce any systemic response when applied i.p. in mice, as assessed by TNF-α serum levels, pulmonary neutrophil recruitment, and lethality when cojected with D-Gal.

In contrast to our findings, current reports in the literature suggest that pancreatic elastase stimulates TNF-α release from murine macrophages, induces elevated TNF-α levels and pulmonary neutrophil infiltration, and causes lethality in the D-Gal model after i.p. application in mice (8, 10, 11, 17). All elastase preparations used in these previous studies were exclusively obtained from one source, pancreatic elastase from Sigma-Aldrich. EU are measured with the LAL assay, which detects the biological activity of an endotoxin. Depending on the source of endotoxin, the conversion from EU to nanograms of LPS will vary. Usually 1 ng of LPS corresponds to ~2–10 EU. We measured an endotoxin content of ~900–4000 EU/U elastase from different preparations from Sigma-Aldrich. This is a large amount, because 1 ng/ml (corresponding to 1–10 EU/ml) of LPS already induces a strong cytokine response in vitro (27, 28, 30).

In the D-Gal lethality model, we found a lethality of 100% before and 80% after passing the elastase preparation (El-IV-1) through a polymyxin B column, but failed to observe any lethality using the enzymatically active low endotoxin elastase preparation (El-UP). Furthermore, we could not observe elevated TNF-α serum levels after i.p. application of El-UP. In conjunction with previously published results, our data show that the polymyxin B agarose column removes most, but not all, endotoxin from protein.

**Discussion**

In this study we demonstrate that pancreatic elastase free of contamination fails to activate murine macrophages to release TNF-α and to cause proinflammatory effects by TNF-α release in vivo, as suggested previously (8, 10, 11, 17). We could also show that the TNF-α-inducing activity of three different elastase preparations was entirely due to contaminating endotoxin. Our conclusions are based on the following findings. 1) Highly purified El-UP was unable to degrade IκBα, induce NF-κB binding activity, or stimulate TNF-α release from RAW 264.7 macrophages and BMDM. 2) Contaminated pancreatic elastase preparations (El-IV) caused degradation of IκBα proteins, induction of NF-κB binding activity, and stimulation of RAW 264.7 macrophages to release large amounts of TNF-α comparable to LPS. 3) Preincubation of the medium with polymyxin B attenuated degradation of IκBα and NF-κB binding activity and eliminated the TNF-α-inducing activity of the contaminated elastase (El-IV). 4) The failure of the low endotoxin (El-UP) preparation to induce TNF-α was not due to enzymatic inactivity, because all enzymes used had comparable enzymatic activities. 5) Low endotoxin elastase failed to induce any systemic response when applied i.p. in mice, as assessed by TNF-α serum levels, pulmonary neutrophil recruitment, and lethality when cojected with D-Gal.

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**Discussion**

In this study we demonstrate that pancreatic elastase free of contamination fails to activate murine macrophages to release TNF-α and to cause proinflammatory effects by TNF-α release in vivo, as suggested previously (8, 10, 11, 17). We could also show that the TNF-α-inducing activity of three different elastase preparations was entirely due to contaminating endotoxin. Our conclusions are based on the following findings. 1) Highly purified El-UP was unable to degrade IκBα, induce NF-κB binding activity, or stimulate TNF-α release from RAW 264.7 macrophages and BMDM. 2) Contaminated pancreatic elastase preparations (El-IV) caused degradation of IκBα proteins, induction of NF-κB binding activity, and stimulation of RAW 264.7 macrophages to release large amounts of TNF-α comparable to LPS. 3) Preincubation of the medium with polymyxin B attenuated degradation of IκBα and NF-κB binding activity and eliminated the TNF-α-inducing activity of the contaminated elastase (El-IV). 4) The failure of the low endotoxin (El-UP) preparation to induce TNF-α was not due to enzymatic inactivity, because all enzymes used had comparable enzymatic activities. 5) Low endotoxin elastase failed to induce any systemic response when applied i.p. in mice, as assessed by TNF-α serum levels, pulmonary neutrophil recruitment, and lethality when cojected with D-Gal.

In contrast to our findings, current reports in the literature suggest that pancreatic elastase stimulates TNF-α release from murine macrophages, induces elevated TNF-α levels and pulmonary neutrophil infiltration, and causes lethality in the D-Gal model after i.p. application in mice (8, 10, 11, 17). All elastase preparations used in these previous studies were exclusively obtained from one source, pancreatic elastase from Sigma-Aldrich. EU are measured with the LAL assay, which detects the biological activity of an endotoxin. Depending on the source of endotoxin, the conversion from EU to nanograms of LPS will vary. Usually 1 ng of LPS corresponds to ~2–10 EU. We measured an endotoxin content of ~900–4000 EU/U elastase from different preparations from Sigma-Aldrich. This is a large amount, because 1 ng/ml (corresponding to 1–10 EU/ml) of LPS already induces a strong cytokine response in vitro (27, 28, 30).

In the D-Gal lethality model, we found a lethality of 100% before and 80% after passing the elastase preparation (El-IV-1) through a polymyxin B column, but failed to observe any lethality using the enzymatically active low endotoxin elastase preparation (El-UP). Furthermore, we could not observe elevated TNF-α serum levels after i.p. application of El-UP. In conjunction with previously published results, our data show that the polymyxin B agarose column removes most, but not all, endotoxin from protein.

**Discussion**

In this study we demonstrate that pancreatic elastase free of contamination fails to activate murine macrophages to release TNF-α...
from the breakdown of endogenous ECM (18–24, 40). They all
Hsp70, Hsp gp96, oligosaccharides, and polysaccharide fragments
infection an inflammatory response for the purpose of tissue repair
involvement of pancreatic elastase in systemic inflammatory re-
Therefore, the present study in the El-IV-1 preparation was 295 ng LPS/U elastase; therefore,
endotoxin content present in the Hsp70 preparation after incubation for 60 min on the polymyxin B column. Nevertheless, considering the large amount of endotoxin present in all elastase preparations from Sigma-Aldrich and the minute amounts needed to evoke respon-
s, even removing 99% of the endotoxin (neglecting elastase protein recovery), sufficient endotoxin would be left to confound results, especially when LPS-sensitizing models are used. The D-Gal lethality model sensitizes mice to the lethal effects of endotoxin, >100,000-fold with an LD₅₀ of 1–5 ng of LPS for sensitized mice (35, 36). The calculated equivalent LPS activity in the present study in the El-IV-1 preparation was 295 ng LPS/U elastase; therefore, >99.7% of the endotoxin would have to be removed from a protein solution containing 1.5 U of elastase to obtain an endotoxin content below the LD₅₀ in D-Gal-sensitized mice.

Only two of the previous reports specified the pancreatic elastase preparation from Sigma-Aldrich used in their studies, elastase type IV (11, 17). Johnson et al. (17) routinely passed the elastase type IV preparation they used through a polymyxin B column to remove possible endotoxin contamination before conjugation with D-Gal in mice. They observed a lethality of 50% with the El-IV preparation. They found that the lethal effect of polymyxin B-purified elastase in the D-Gal model was sensitive to boiling and partly sensitive to preincubation with an elastase inhibitor (17). One explanation for this might be that LPS is heat sensitive at low concentrations, as shown by Gao and Tsan (27, 28). The finding that preincubation with an elastase inhibitor attenuated lethality could be explained by the observations of Wielock et al. (37) that pretreatment of D-Gal-sensitized mice with serine protease inhib-
itors in general protects from lethality after LPS treatment. Fur-
thermore, recent data demonstrate that serine protease inhibitors are able to inhibit LPS-mediated activation of monocytes in vitro (38). The work of Pastor et al. (39) also questions the concept of elastase playing a significant part in the state of SAP by cleaving heparan sulfate from extracellular matrix to trigger SIRS by activating innate immunity via TLR4 signaling. They found no dif-
ference in the severity of pancreatitis and pancreatitis-associated lung injury after induction of cerulein pancreatitis in WT and TLR4⁻/⁻ mice (39). Our results do not necessarily rule out an involvement of pancreatic elastase in systemic inflammatory re-
sponse observed in SAP. It seems reasonable that in the absence of infection an inflammatory response for the purpose of tissue repair is triggered upon destruction of ECM by pancreatic enzymes. However, whether a state of systemic inflammatory response oc-
curs remains questionable.

All putative endogenous ligands of the TLRs proposed to date seem to signal through TLR4 or TLR2. They include Hsp60, Hsp70, Hsp gp96, oligosaccharides, and polysaccharide fragments from the breakdown of endogenous ECM (18–24, 40). They all have been shown to activate immune cells only at very high con-
centrations, which is in sharp contrast to the minute amounts of

LPS (<1 ng/ml) required to activate TLR2/4-mediated signaling. For instance, pancreatic elastase has been used in concentrations >100 μg protein/ml in previous studies to demonstrate its mac-
rophage-activating and systemic inflammatory qualities (8, 9, 17).

This fact raises the suspicion that these putative TLR2/4 ligands might be contaminated with LPS or bacterial lipopolysaccharides, as previously demonstrated for Hsp60 and Hsp70 (27–30). Whether other endogenous host molecules can be true ligands for TLR2/4 or are the result of contaminated LPS or bacterial lipopolysaccharide is the subject of current debate (41–44). Although our data cannot rule out indirect proinflammatory actions of pancreatic elastase, they again underline the absolute necessity of using essentially endo-
toxin-free enzyme preparations when efforts are undertaken to study possible proinflammatory effects on innate immunity.

Disclosures
The authors have no financial conflict of interest.

References


