Biochemical Pharmacology, Vol. 40, No. 7, pp. 1601–1603, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

IN VIVO EVIDENCE FOR PROTEASE-CATALYSED MECHANISM PROVIDING BIOACTIVE TUMOR NECROSIS FACTOR α

MARCUS NIEHÖRSTER, GISA TIEGS, ULRICH F. SCHADE* and ALBRECHT WENDEL†
Faculty of Biology, University of Konstanz, P.O. Box 5560, D-7750 Konstanz, Federal Republic of Germany

(*Received* 3 *October* 1989; *accepted* 11 *May* 1990)

Abstract—Mice pretreated by intravenous injection of 42 mg/kg of the serine protease inhibitor α_1-antitrypsin prior to a hepatotoxic dose of D-galactosamine/lipopolysaccharide (GalN/LPS) were fully protected against hepatitis. Pretreatment with α_1-antitrypsin with doses up to 300 mg/kg at different times failed to protect galactosamine sensitized animals against tumor necrosis factor α (TNFα)-induced hepatitis. No bioactive TNFα was detectable in serum of mice protected against GalN/LPS-induced hepatitis by pretreatment with α_1-antitrypsin. In contrast, abundant amounts of TNF were found in sera of GalN/LPS-treated control animals. It is concluded that a serine protease sensitive to α_1-antitrypsin provides bioactive TNFα by proteolytic cleavage of a TNFα precursor.

The involvement of proteases of leukocytic origin is believed to play a pivotal role in inflammation [1] and shock [2]. It is under debate, however, whether this process is a passive response or whether it is actively involved in mediating cytotoxicity. We studied this question in an *in vivo* model of acute inflammation, i.e. fulminant hepatitis in GalN-sensitized mice which is induced by administration of either lipopolysaccharide (LPS,‡ endotoxin) [3] or its toxic mediator, tumor necrosis factor α (TNFα) [4, 5].

Challenge of animals with either agent, i.e. LPS or TNFα, leads to a common pathophysiological endpoint. This is reached either by initiation via LPS at the very beginning of a deleterious cascade, or triggered in a terminal state of the inflammatory response in the case of TNFα. Therefore, comparative intervention studies with an antiprotease in the two models and determination of TNF in sera of these mice seem suitable to assess the qualitative contribution of extracellular protease activity to inflammation, as well as to deduce the chronological sequence of events.

MATERIALS AND METHODS

Animals and experimental design. Male NMRI mice (30 g, from Thomae, Biberach, F.R.G.) were kept at least 1 week under controlled conditions (27°, 55% humidity, 12 hr day/night rhythm) and fed a standard laboratory diet (Altromin C 1000). Twelve hours prior to the experiments, food was withdrawn. The animals received injections at 8.00 a.m. as detailed in the legends to Tables 1 and 2. Blood for determination of TNF was obtained by eye puncture one hour after LPS-administration. At 5.00 p.m., the mice were killed and blood was withdrawn as described in Ref. 5.

Enzyme assay procedures. Plasma transaminases and sorbitol dehydrogenase activities were determined according to Ref. 6.

TNF assay. TNF in serum of mice was determined essentially as described in Ref. 7. Fibrosarcoma cells (WEHI 164 clone 13, kindly provided by Dr T. Espevik, Trondheim, Norway) at a concentration of 2×10^4 cells/100 μL were incubated with serially diluted test samples in 96-well, flat-bottomed microtiter plates (37°, 5% CO_2, 18 hr). The supernatants were removed and MTT (5 mg/mL, 1/10 v/v) was added for determination of cytotoxicity.

After incubation for 4 hr at 37° the dye was removed, cells were lysed by addition of 100 μL isopropanol/5% formic acid. Plates were read on a SLT EAR 400 microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The titer of TNF is expressed in units/mL and is defined as the reciprocal of the dilution necessary to cause death of 50% of the cells.

Reagents. D-Galactosamine · HCl was purchased from Serva (Heidelberg, F.R.G.). Murine recombinant TNFα was a generous gift by Dr G. R. Adolf, Ernst-Boehringer-Institut, Vienna, Austria. Human α_1-antitrypsin was provided by Dr R. Neumann, Troponwerke, Köln, F.R.G. Lipopolysaccharide *Salmonella abortus* equi was from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Statistics. The results were analysed according to Student's *t*-test and are given as mean values ± SE. $P \leq 0.05$ was considered to be significant.

RESULTS

Treatment of GalN-sensitized animals with either LPS or TNFα led to dramatic increase of plasma

* Forschungsinstitut Borstel, D-2061 Borstel, F.R.G.

† To whom correspondence should be addressed.

‡ Abbreviations: α_1-AT, α_1-antitrypsin; ALT, alanine amino transferase; GalN, D-galactosamine; LPS, Lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TNFα, tumor necrosis factor α.

Table 1. Protection by the protease-inhibitor α_1-antitrypsin (α_1-AT) against GalN/LPS-, but not against GalN/TNFα-induced hepatitis in mice

	GalN/LPS			GalN/TNFα		
Pretreatment	ALT [units/L]†	N	M	ALT [units/L]†	N	M
Hepatic injury control	2708 ± 1004	4	1	2698 ± 1150	5	1
42 mg/kg α_1-AT	26 ± 6*	4	0	4293 ± 1423	6	1
300 mg/kg α_1-AT	34 ± 12*	4	0	4790 ± 1957	6	3

* $P \leq 0.05$ vs hepatic injury control.

† Plasma ALT was determined 8 hr after administration of LPS and TNFα, respectively, analogous data were obtained by determination of aspartate amino transferase and sorbitol dehydrogenase, doses and application routes: GalN: 700 mg/kg i.p., LPS: 5 μg/kg i.p. simultaneous with GalN, TNFα: 15 μg/kg i.v. 1 hr after GalN, α_1-AT: i.v., 30 min prior to GalN.

N = number of animals per group, M = number of animals which died within 8 hr. Values are means ± SE.

Table 2. TNF-levels in serum of mice protected against GalN/LPS-induced hepatitis by pretreatment with α_1-antitrypsin

Pretreatment	ALT [units/L]†	TNF [units/mL]‡	N	M
Hepatic injury control	1716 ± 290	560 ± 145	6	3
42 mg/kg α_1-AT	114 ± 22*	ND§	4	0
Solvent control‖	2386 ± 414	640 ± 166	4	1

* $P \leq 0.01$ vs hepatic injury control.

† Plasma ALT was determined 8 hr after administration of LPS, analogous data were obtained by determination of aspartate amino transferase and sorbitol dehydrogenase.

‡ Serum TNF was determined 1 hr after administration of LPS.

§ Detection limit: 20 units/mL.

‖ Solvent control group animals were treated with α_1-AT-solvent (phosphate buffered saline) 30 min prior to GalN/LPS, doses and application routes: GalN (700 mg/kg) was administered simultaneously with 5 μg/kg LPS i.p., α_1-AT (42 mg/kg) was applicated i.v. 30 min prior to GalN/LPS.

N = number of animals per group, M = number of animals which died within 8 hr. Values are means ± SE.

ALT 8 hr after challenge. When LPS-intoxicated animals had been pretreated with α_1-antitrypsin, they were fully protected against hepatitis and none of them died within 8 hr (Table 1, left column). In contrast, an analogous pretreatment with the anti-protease, even in a much higher dose, failed to have any significant effect on the extent of hepatitis and mortality induced by administration of TNFα (Table 1, right column). These results show that successful intervention by intravenous administration of anti-protease is possible before a LPS-challenge but does not prevent TNFα-induced hepatitis. The findings suggest that the involvement of protease activity in the inflammatory process takes place prior to TNFα cytotoxicity.

Therefore, we were interested to know whether protective pretreatment with α_1-antitrypsin had affected systematic TNF-levels following a LPS-challenge. The data in Table 2 show that, within the limits of our very sensitive bioassay, no TNF was detectable in serum of mice protected against GalN/LPS-induced hepatitis by administration of α_1-antitrypsin. In the positive control experiment, high amounts of serum TNF and concomitant hepatic injury as assessed by plasma transaminase-levels was observed in GalN/LPS-treated mice.

These findings indicate that the systematic presence of TNF upon an LPS-stimulus requires a serine protease activity sensitive to α_1-antitrypsin.

DISCUSSION

The central role of proteases in the inflammatory reaction is known since a long time. It remained open however, whether protease activity released from stimulated leukocytes would proteolytically attack cellular structures and thus lead to impairment of physiological functions in a direct way, or whether their effect is a specific contribution to a programmed pathophysiological sequence. Our findings clearly favour the latter view.

The results in Table 1 show on the one hand that the deleterious activity of TNFα, once present, is independent of elastase activity. On the other hand, the results in Table 2 demonstrate that the disposal of active TNF when triggered by LPS requires a protease-dependent step. These findings might be explained as follows:

In vitro experiments showed that a membrane-bound form of TNFα exists in cells known to release TNFα [8]. Independently it was reported that TNFα-release upon LPS-stimulus in mono-nuclear cells was

blocked by a serine protease inhibitor [9]. Moreover, the N-terminus of the soluble bioactive form of TNFα is known to contain suitable amino acids for cleavage by elastase (valine in human TNFα, leucine in mouse TNFα) [10, 11].

Based on these findings we presume that LPS might induce the synthesis of a known 26 kD TNFα precursor which is released as a soluble, bioactive, 17 kD form following proteolytic cleavage. Our experiments provide strong evidence that this is actually the case *in vivo*.

It seems, therefore, that proteolytic attack on cellular structures does not represent an unspecified cytotoxic mechanism in the two models studied. It rather appears that protease release plays a specific role in providing active mediator upstream from an ultimate hepatotoxic action of TNFα.

Acknowledgements—This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 156, Grant We 686/12). We are indebted to Dr G. R. Adolf and Dr Neumann for generously providing TNFα and α_1-antitrypsin, respectively.

REFERENCES

1. Weiss SJ, Tissue destruction by neutrophils, mechanisms of disease. *N Engl J Med* **320**: 365–376, 1989.
2. Jochum M and Fritz H, Pathobiochemical mechanisms in inflammation. In: *Immune Consequences of Trauma, Shock and Sepsis*, pp. 165–172. Springer, Berlin, 1989.
3. Galanos C, Freudenberg MA and Reutter W, Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc. Natl Acad Sci USA* **76**: 5939, 1979.
4. Lehmann V, Freudenberg MA and Galanos C, Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J Exp Med* **165**: 657–663, 1987.
5. Tiegs G, Wolter M and Wendel A, Tumor necrosis factor is a terminal mediator in galactosamine/endotoxin-induced hepatitis in mice. *Biochem Pharmacol* **38**: 627–631, 1989.
6. Bergmeyer H-U, *Methods of Enzymatic Analysis*, 3rd Edn. Verlag Chemie, Wienheim, 1984.
7. Espevik T and Nissen-Meyer J, A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor tumor necrosis factor from human monocytes. *J Immunol Methods* **95**: 99–105, 1986.
8. Kriegler M, Perez C, DeFay K, Albert I and Lu SD, A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* **53**: 45–53, 1988.
9. Scuderi P, Suppression of human leukocyte tumor necrosis factor secretion by the serine protease inhibitor *p*-toluene-sulfonyl-L-arginine methyl ester (TAME). *J Immunol* **143**: 168–173, 1989.
10. Webb D and Goeddel D, *Lymphokines*, Vol. 13. Academic Press, San Diego, 1987.
11. Powers JC, Gupton BF, Harley AD, Nishino N and Whitley RJ, Specificity of porcine pancreas elastase, human leukocyte elastase and cathepsin G. *Biochem Biophys Acta* **485**: 156–166, 1977.