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TIME COURSE OF IL-6 AND TNFα RELEASE DURING ENDOTOXIN-INDUCED ENDOTOXIN TOLERANCE IN RATS

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Abstract—Development of endotoxin tolerance in rats induced by repeated application of low dosages of endotoxin is associated with repeatable IL-6 formation, reversible drop in white blood cells, pronounced consumption of platelets, gradual formation of α_2M as an example of acute phase proteins, and flattening TNFα formation. In the status of full tolerance the TNFα release is completely eliminated. Inhibition of TNFα biosynthesis and induction of acute phase protein formation by IL-6 are discussed as possible factors in the development of endotoxin tolerance.

Bacterial lipopolysaccharides (LPS‖), also addressed to as endotoxins, are well documented to mimic the phenomena of septicemic and polytraumatic shock in various species and are therefore also considered the primary inducers of the cascade of pathogenic events leading to the often lethal outcome in these serious clinical conditions. Given at sub-lethal dosages, however, LPS is known to induce a state of tolerance against lethal dosages of the same or other endotoxins [1]. This phenomenon, though known for decades already, lacks any conclusive explanation.

The difficulties associated with the elucidation of the mechanisms responsible for endotoxin tolerance are easily envisaged if the complexity of endotoxemic shock itself is considered. Pathophysiologically, septic or polytraumatic shock may be simply characterized by a severely disturbed microcirculation of vital organs usually combined with impaired gas exchange due to lung edema. The underlying pathobiochemical alterations comprise a dramatic increase of eicosanoid production [2] and PAF acether [3], release of cytokines, in particular of IL-1, IL-6, and TNF [4–6], formation of oxygen-centered free radicals from polymorphonucleocytes and macrophages [7], release of leukocyte elastase [8] accompanied with oxidative inactivation of serine protease inhibitors, activation of the plasmatic coagulation cascade as well as of fibrinolysis [9], complement activation [10], expression of adhesive proteins on phagocytes and of their endothelial receptors [11].

More recently it became evident that TNFα is a necessary though not sufficient effector of lethality in endotoxemia [12, 13]. On the other hand IL-6 has been suggested to present its physiological counterpart in dampening the effect and/or formation of TNFα. IL-6 is known to induce the formation of acute phase proteins which comprise protease inhibitors [14] supposed to prevent tissue destruction e.g. by leukocyte elastase [8].

Correspondingly, injected IL-6 has been demonstrated to induce hyporesponsiveness to endotoxin in mice [15]. It has further been demonstrated that IL-6 inhibits the release of TNFα in human U937 cells and monocytes *in vitro* and in mice *in vivo* [16].

It therefore appeared revealing to measure the formation of TNFα and IL-6 in rats during tolerance induction by low dose endotoxin and after a massive dose of endotoxin provoking lethal shock in susceptible animals.

METHODS

Experimental protocol. Male Sprague–Dawley SPF rats, 200–300 g (breeder: Hagemann, Frankfurt, F.R.G.) were housed under standard conditions. Four days before endotoxin infusion polyethylene catheters (PP 10 connected with PP 20) were implanted in the left jugular vein under ketamin hydrochloride (Ketanest®, 100 mg/kg i.p.) and xylazine (Rompun®, 16 mg/kg i.p.) anesthesia. Two days before the endotoxin infusion 1 mg/kg LPS (*E. coli* 0127:B8 purchased from the Sigma Chemical Co., St Louis, MO, U.S.A. dissolved in 1 mL 0.9% NaCl) was injected into the tail vein. Twenty four hours later another dose of LPS (2.5 mg/kg) was intravenously injected. The control group received the same amount of i.v. NaCl. Forty eight hours after the first LPS injection an endotoxin shock was induced by intravenous infusion of 50 mg/kg LPS over 4 hr. In survival studies the animals were observed up to 7 days after the start of the endotoxin infusion. Blood was obtained either by retro-orbital plexus puncture or via the venous jugular catheter. For the investigation of leukocyte sticking in the

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‖ Abbreviations: α_2M, α_2 Macroglobulin; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; (r)IL-6, (recombinant) interleukin-6; and WBC, total white blood cells.

lungs the animals were killed 6 hr after shock induction. Because of the large number of blood samples needed two groups of animals were used in each experiment, one for collecting blood during the induction of the tolerance (N = 6), the other one for taking samples during the endotoxic shock and for the determination of the survival rate (N = 9).

α_2M determination. α_2M-Macroglobulin serum concentrations were determined by rocket immunoelectrophoresis according to Laurell [17]. The agarose solutions contained 0.66% of the antisera raised in rabbits. Purified rat α_2M was used as a standard. The rat sera were diluted 1:20.

IL-6 bioassay. IL-6 was determined using the B9 cell proliferation assay as described by Aarden *et al.* [18]. IL-6-dependent B9 cells from a murine hybridoma cell line were cultured with serum samples (heat-inactivated at 56° for 30 min) for 72 hr. The proliferation of the cells was measured by [^{3}H]thymidine incorporation. One unit/mL is defined as the amount of IL-6 that caused a 50% decrease of maximal [^{3}H]thymidine incorporation. rhIL-6 (prepared from recombinant *E. coli*; specific activity of approximately 3.8×10^8 units/mg in B9-cell test, kindly provided by T. Kishimoto and T. Hirano, Osaka, Japan) is used as an internal standard. The specificity of the assay has been reported previously [19].

TNFα assay. Cytolytic TNF activity was determined by means of a TNFα-sensitive WEHI 164 clone 13 cell line [20]. Briefly, 2×10^4 cells/100 μL suspended in RPMI, 10% fetal calf serum were plated on 96 well microliter plates and incubated for 4 hr at 37° to yield a monolayer. A serial dilution of each serum sample was incubated with the WEHI cells for 18 hr at 37°. To measure the vitality of the cells 20 μL of MTT were added to each well and incubated for 4 hr at 37°. The reduction of the pigment was measured in an ELISA reader at 570 nm. One unit/mL is defined as the dilution of the sample that causes a 50% reduction of maximum vitality in the assay.

Myeloperoxidase assay. Six hours after the beginning of the endotoxin infusion some animals were killed by intravenous pentobarbitone overdosage (2 mg/kg i.v.); the anterior lobe of left lung was excised and immediately frozen in liquid nitrogen. The lung tissue was homogenized exactly as described before [21]. The myeloperoxidase activity was measured spectrophotometrically with tetramethyl-benzidine dihydrochloride (Fa. Aldrich) as a chromogenic hydrogen donor essentially according to [22]. The technique of Suzuki *et al.* [22] was modified by using microtiter plates and a different buffer system (Na citrate buffer, pH 4.5). One unit of MPO activity is defined as the amount degrading 1 μmol peroxide per minute at 25°. The results are expressed as units/mg lung tissue.

Blood cell determination. For blood cell determination blood was collected into EDTA-coated vials. Samples for the determination of white blood cells, platelets, and hematocrit (20 μl) were diluted in a Sysmex® dual dilutor DD-100 and injected into a Sysmex® microcell counter (Toa Medical Electronics, Kobe, Japan).

Statistics. All data are expressed as mean ± SEM. For comparison of the different time values and groups Kruskal–Wallis test was used. Differences were calculated by Student's *t*-test. Chi square test was used to calculate the statistics of the survival rate.

RESULTS

As shown in Fig. 1 a well tolerated dose of 1 mg/kg LPS induced a dramatic increase of circulating IL-6 within 2 hr which decreased to a still significantly elevated level at 22 hr. A second dose of LPS (2.5 mg/kg) given in the 24th hour led to a similar pattern of IL-6 release. The levels achieved, however, were slightly lower despite the increased LPS dose. The shock-inducing dose of LPS (50 mg/kg) administered 48 hr after the first dose again elicited the same IL-6 response (Fig. 1, filled columns). In contrast, in the animals not pretreated the same endotoxin dose (empty columns) induced an about 10 times higher IL-6 release ($P < 0.001$).

All pretreated animals survived for 7 days, whereas expectedly all endotoxin-naive animals died upon 50 mg/kg LPS within 24 hr (Fig. 2). The low tolerance-inducing LPS dose already induced a sustained drop in platelets (Fig. 3). The corresponding fall in total white blood cells (Fig. 4) was reversible even after the shock-inducing dosages in the tolerant animals, while the white blood cells did not recover in the non-tolerant animals. Post mortem measured myeloperoxidase activity in the lungs supported the interpretation that a significantly higher percentage of polymorphonucleocytes remained sticking in the pulmonary micro-circulation of the rats suffering from endotoxin shock as compared to the tolerant rats killed at the same time (4.705 ± 0.506 units/mg in the LPS-tolerant group versus 6.178 ± 0.538 units/mg lung tissue in the untreated rats).

As a typical response to IL-6 the rise in α_2 macroglobulin was determined by immunological techniques during tolerance development and shock induction. As shown in Fig. 5, the α_2M increase was delayed considerably in respect to the rise in IL-6. Significantly increased α_2M values were not detected before 26 hr after the first endotoxin dose. However, at the time when full tolerance was consistently reached the α_2M levels were markedly elevated. The non-tolerant animals had slightly elevated α_2M levels from the beginning due to the stress associated with implantation of the jugular vein catheter; their α_2M levels did however not change within the shock period of 6 hr.

The non-fatal dose of 1 mg/kg LPS induced a dramatic rise of TNFα from undetectable levels to about 20,000 units per mL which are not significantly lower than the 50,000 units observed after the lethal dose of 50 mg/kg LPS (Fig. 6). This only small difference of the TNFα response may be surprising, although these data confirm the observation of Feuerstein *et al.* [12] that the LD_{50} of endotoxin is not at all paralleled by its ED_{50} in terms of TNFα induction. Even after the low dosage of LPS elevated TNFα levels can be clearly measured for at least 24 hr.

The time course of TNFα plasma levels appears consistent with the data of Waage [23]: Our maximum levels (around 20,000 units/mL 2 hr after 1 mg/kg

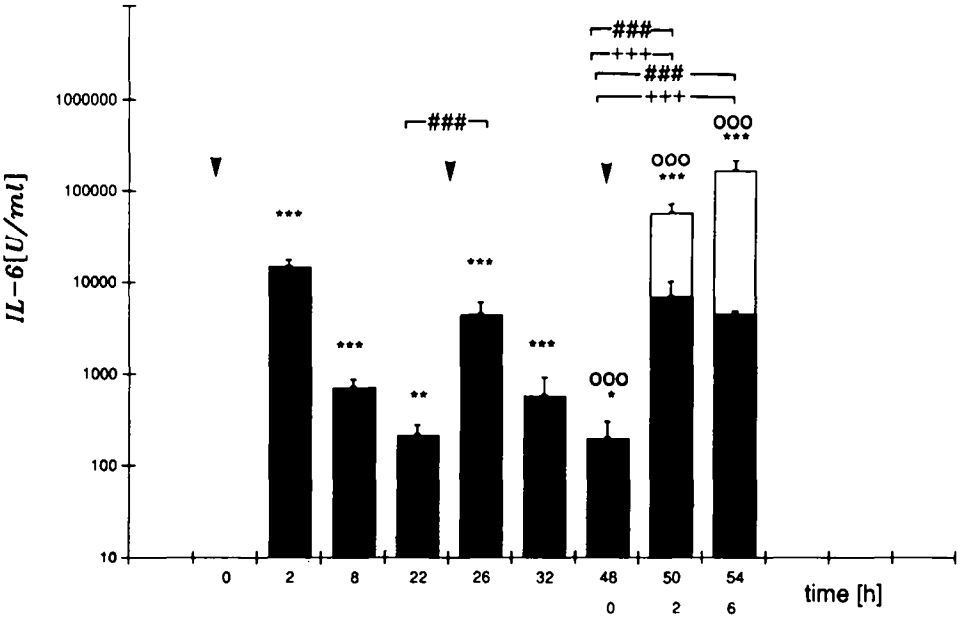

Fig. 1. Interleukin-6 levels in blood of rats exposed to endotoxin (arrows, V) to induce endotoxin tolerance or overt endotoxemic shock. Six animals per group were investigated. At time zero (first arrow) 1 mg/kg endotoxin was applied. The second dose of 2.5 mg/kg and the challenging dose of 50 mg/kg were applied at 24 and 48 hr, respectively. Shadowed columns show the data of the animals subjected to the tolerance-inducing scheme. Void columns show data of endotoxin-naive animals exposed to the challenging dose at 48 hr only. Asterisks (*) indicate significant differences versus pre-exposure in the group of animals rendered tolerant, italic crosses (#) express significant differences between the time values indicated by brackets in the endotoxin-tolerant group; crosses (+) indicate significant differences versus pre-exposure values as shown by brackets in endotoxin-naive animals; circles (O) indicate group differences. One, two and three symbols mean $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

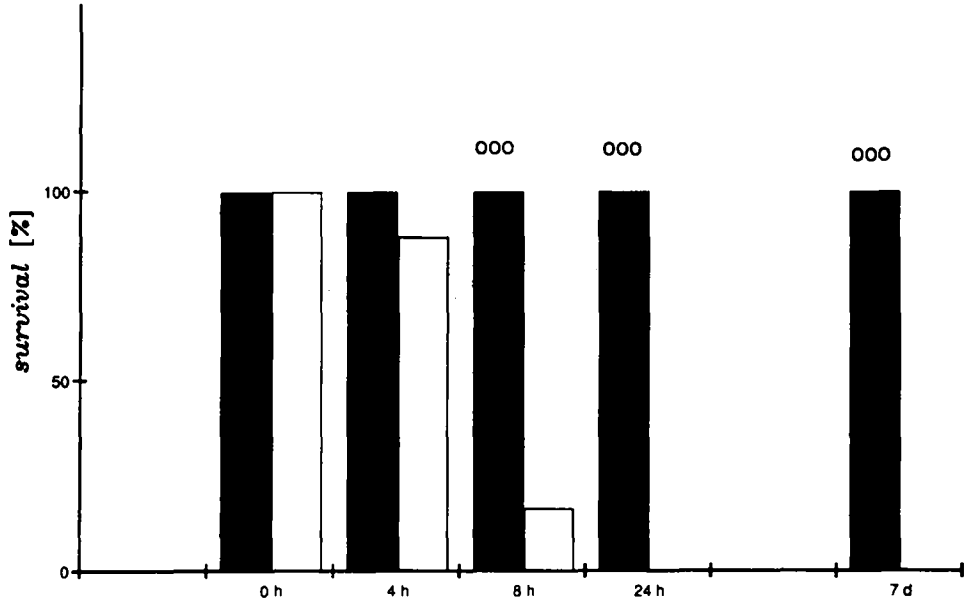

Fig. 2. Seven-day survival of tolerant (filled columns) and non-tolerant rats (empty columns) challenged with 50 mg/kg endotoxin. Twelve animals per group were investigated. For further explanation of symbols see Fig. 1.

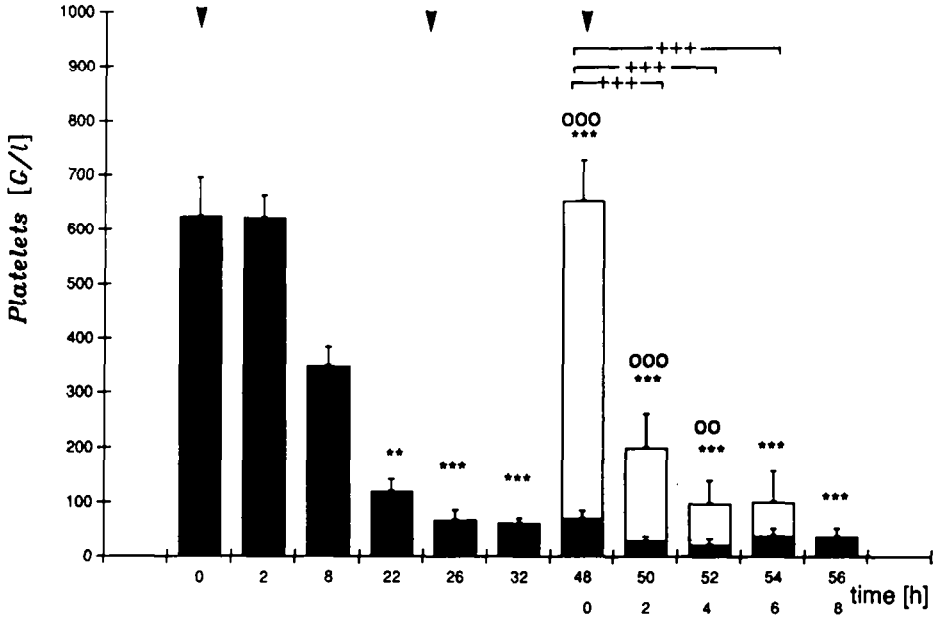

Fig. 3. Decline of blood platelet count during endotoxin-induced endotoxin tolerance and endotoxemic shock in rats. Nine animals per group were investigated. For explanation of symbols see Fig. 1.

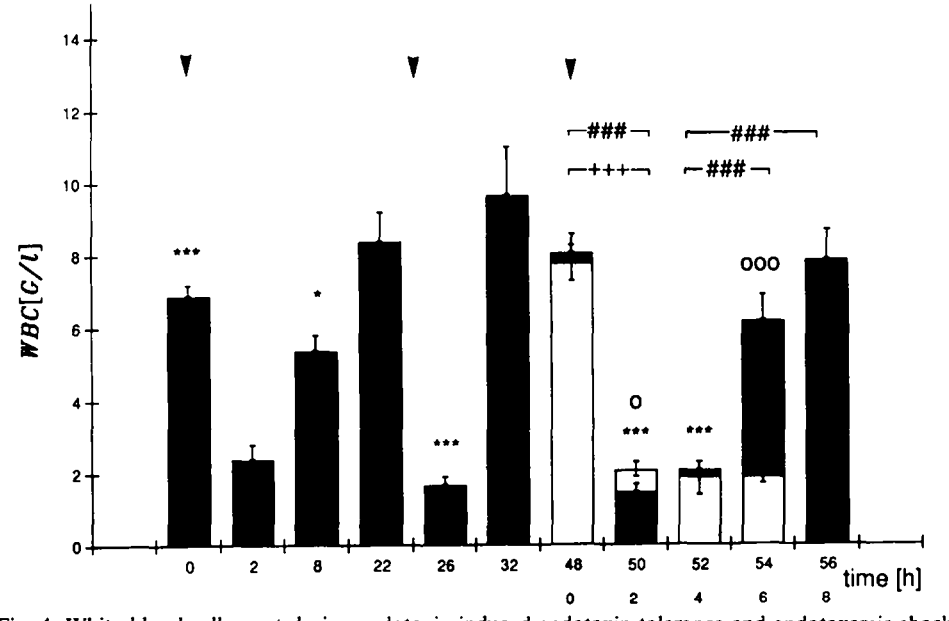

Fig. 4. White blood cell count during endotoxin-induced endotoxin tolerance and endotoxemic shock in rats. Six animals per group were investigated. For explanation of symbols see Fig. 1.

LPS) are slightly below the peak levels reported by Waage [23] to occur 1 hr after LPS application. Also, the plasma concentrations after several hours are consistently measured near 1000 units/mL in rats [23], whereas in rabbits [24, 25], mice [26] and man [27] TNFα appears to decline faster.

The second low dose of endotoxin only yielded a small increment on top of the already elevated TNFα level. Forty eight hours later the shock-inducing dosage of 50 mg/kg LPS did not induce any further rise in TNFα in the tolerant rats, whereas the same dose in endotoxin-naive rats, as already mentioned, lead to 50,000 units TNFα/mL after 2 hr and killed all the animals within 8 hr.

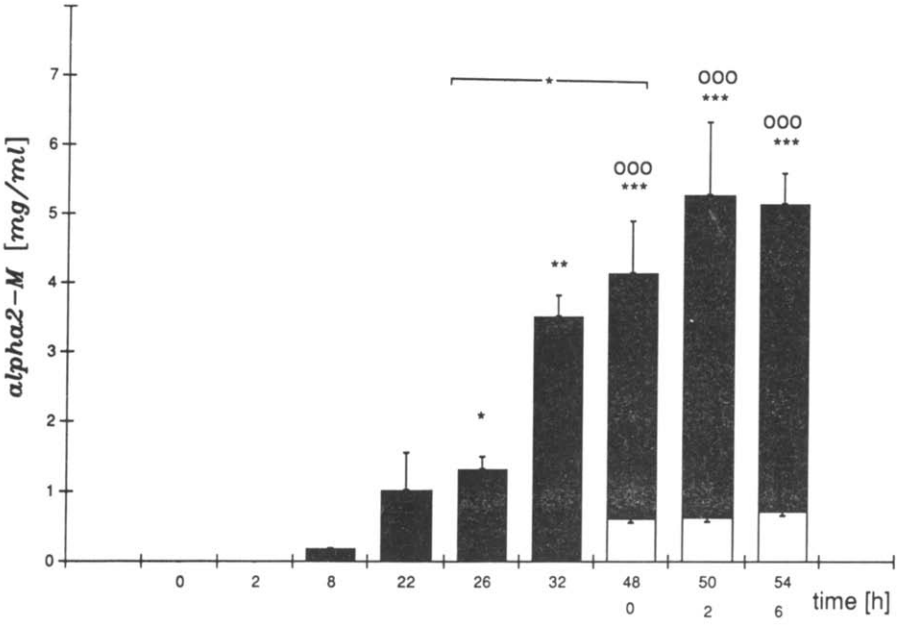

Fig. 5. Levels of α_2 macroglobulin in the blood of rats during endotoxin-induced endotoxin tolerance and endotoxemic shock. Six animals per group were investigated. For explanation of symbols see Fig. 1.

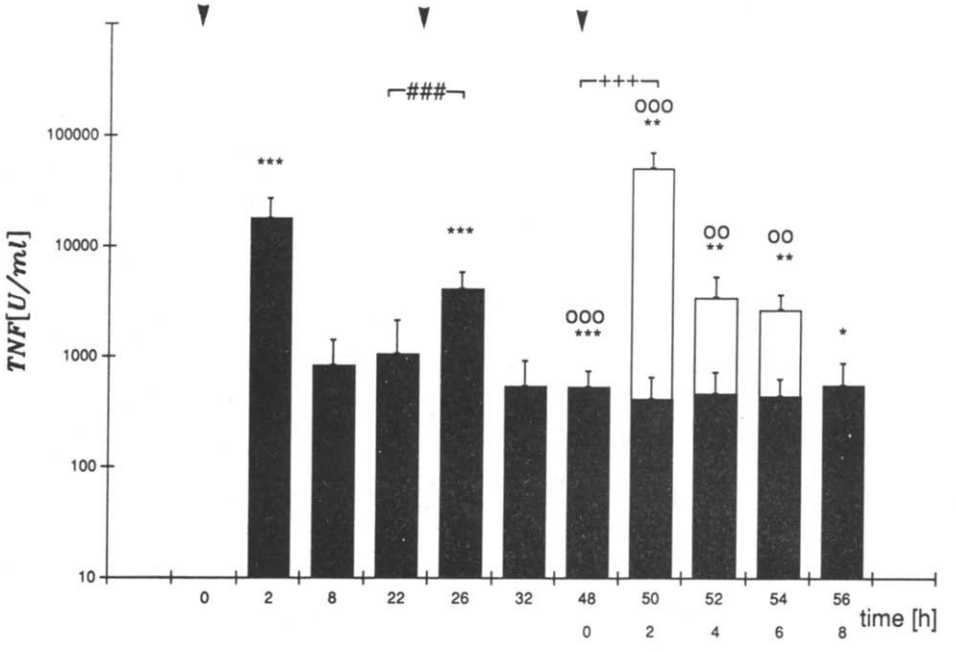

Fig. 6. Levels of TNFα in the blood of rats during endotoxin-induced endotoxin tolerance and endotoxemic shock. Six animals were investigated during tolerance development. For the challenge period (starting at $t = 48$ or 0 hr respectively) nine animals per group were used. For explanation of symbols see Fig. 1.

DISCUSSION

The study of shock-related phenomena in rats has often been criticized with regard to the atypically high endotoxin dosages tolerated by this species. Apart from this peculiarity, however, rats qualitatively respond to endotoxin in a very similar manner as do mice and primates. Identical lipid mediators and cytokines are released upon endotoxin exposure and, physiologically, the endotoxin

response in rats is characterized by fever, disseminated intravascular coagulation, platelet aggregation, white blood cell adhesion, collapse of microcirculation and lung edema etc. as in other species and in patients. Also, experimental strategies to overcome endotoxemic shock or organ failure in animals revealed little difference between rats, mice and primates in terms of efficacy. Prostacyclin analogs prevented endotoxin/galactosamine-induced liver necrosis in mice [28] and improved survival of endotoxin-treated rats [29]. Similarly, superoxide dismutase which scavenges the superoxide radicals released from activated phagocytes proved equally effective in endotoxin-treated mice [28] and rats [30, 31]. Antibodies against TNFα prevented fatal endotoxin-dependent organ failure in mice [32] and baboons [33].

The data obtained with our model of endotoxin-induced endotoxin tolerance are in line with the assumption that TNFα release is an inevitable event leading to the fatal outcome of endotoxin shock. Clearly, the most prominent feature observed is the complete inability of the tolerant animals to respond to endotoxin exposure with TNFα release. This observation essentially agrees with that of Sanchez-Cantu *et al.* [34] who found a blunted TNFα release when tolerant rats were exposed to endotoxin. This phenomenon has also been observed in other species [35] and is attributed to an impaired ability of macrophages to release TNFα [35–37]. Interestingly, Waage [23] describes that the TNFα release upon endotoxin challenge recovers after 7 days which coincides with the disappearance of tolerance [38]. The efficacy of neutralizing antibodies against TNFα in shock suppression observed in a variety of species [32, 33] and the lack of TNFα formation associated with 100% survival of heavily endotoxin-treated rats described here lend further support to the role of TNFα as a prominent autacoid toxin within the realm of shock mediators.

Our data, however, also support the view that TNFα by itself cannot be the only crucial mediator of lethality in endotoxemia. As previously pointed out by Feuerstein *et al.* [12], already well tolerated doses of endotoxin (e.g. 1 mg/kg in our experiments) provoke an almost maximum TNFα response which is hardly statistically differentiated from that to a fatal dose (50 mg/kg). The quantitative differences in the TNFα release observed after 1 mg/kg and 50 mg/kg endotoxin given to naive rats are definitely too small to convincingly explain an all or none difference in survival.

TNFα release in endotoxemia has also been implicated in the induction of intravascular coagulation [39], of consecutive platelet aggregation and of obstructive white blood cell adhesion to the endothelium of the capillary bed. TNFα is known to induce exposure or expression of the integrins CD11b and CD11c on the surface of phagocytes and of the corresponding ligands on the endothelium such as ICAM-1, ELAM-1, VCAM [11] and has therefore been suggested to contribute to the disturbance of the microcirculation, although it has definitely to share this potential with a variety of other cytokines and lipid mediators. Trying to correlate the TNFα release in our experiments with counts of circulating cells we have to face some phenomena which are not easily explained. During tolerance development each TNFα increase upon endotoxin administration leads to a dramatic, though transient fall in circulating white blood cells. The fatal dose of endotoxin given to non-tolerant animals lead to both, extreme TNFα levels and irreversible leukocyte sticking. So far, the role of TNFα in provoking PMN and monocyte adhesiveness appears to be supported by our data. However, the 50 mg/kg dose of endotoxin, which does not trigger any further TNFα release in tolerant rats, leads to the same initial drop in white blood cell count. The only difference detectable between the fully tolerant and susceptible animals consists in the reversibility of the leukopenia. In view of these discrepancies the impact on white blood cell/endothelium interactions ascribed to TNFα based on *in vitro* experiments [11] is not likely to be prominent *in vivo*. The complex effects of endotoxins and related shock mediators described in detail elsewhere [40, 41] are also of questionable relevance to endotoxin pathogenicity, since it has been shown that strategies intervening with the pathogenic effects of endotoxin do not essentially alter the disturbances of the differential blood cell counts [42]. The correlation between TNFα release and disappearance of platelets from the circulation appears similarly vague.

As observed with TNFα, IL-6 is also increased by administration of endotoxin doses not provoking shock. In contrast to the TNFα response, the IL-6 response is however not abrogated during tolerance development. The fully tolerant animals still respond to endotoxin with IL-6 formation, although to a lesser degree than naive animals. The IL-6 levels present during the phase of tolerance development should be high enough to down regulate TNFα formation. The peak values, in fact, exceed the IL-6 concentrations leading to a protracted suppression of LPS-induced TNFα biosynthesis *in vitro* [16]. In this context it appears interesting that the duration of preexposure to IL-6 determined the consecutive suppression of TNFα biosynthesis in the absence of IL-6, whereas IL-6 incubated simultaneously with LPS was less effective in down regulating TNFα biosynthesis [16]. The status of tolerance would thus be a consequence of IL-6-induced inhibition of TNFα biosynthesis, whereas in the non-tolerant status LPS triggers TNFα formation and, as suggested by Fong *et al.* [43], IL-6 release possibly in response to the TNFα peak. If indeed IL-6 were the cytokine responsible for tolerance development, IL-6 formation known to be induced by IL-1 and TNFα [43] could also explain the endotoxin tolerance induced by these cytokines [44] and the phenomenon of mutual cross tolerance.

It may however be hazardous to ascribe the phenomenon of endotoxin tolerance to one single phenomenon. As TNF is certainly not the only killing factor within the cocktail of shock mediators, IL-6-dependent inhibition of TNFα formation is not likely the only self-protective mechanism of the organism. It appears suggestive that the acute phase protein α_2-macroglobulin is induced by low dose endotoxin—

and probably mediated by IL-6 as previously shown [45]—within a time frame similar to that required for tolerance formation. It therefore remains attractive to further investigate the role of α_2M and other acute phase reactants in the context of endotoxin tolerance.

In conclusion, the status of endotoxin tolerance in rats is characterized by a complete abrogation of the TNFα release as a response to endotoxin exposure, whereas the IL-6 response is still maintained. This observation is in accordance with the concept [16] that persistently elevated IL-6 levels lead to a down-regulation of TNFα formation and thereby induce tolerance. Acute phase proteins formed upon release of IL-6 or other cytokines may however contribute to the status of tolerance.

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