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Serum levels of tumor necrosis factor determine the fatal or non-fatal course of endotoxic shock

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1. Summary

The role of tumor necrosis factor alpha (TNFα) in endotoxin-induced shock was investigated in pigs receiving 5 μg kg^{-1} of *Escherichia coli* endotoxin (LPS) during 60 min of continuous infusion into the superior mesenteric artery. LPS concentration in aortic plasma, as determined by a chromogenic *Limulus* amoebocyte lysate (LAL) test, reached a peak of approximately 1000 ng l^{-1} during LPS infusion, and declined rapidly after discontinuation of the infusion. Serum TNF levels were determined by a bioassay using the L929 murine transformed fibroblast line. Eight of the 17 animals infused with LPS died within 30 min after beginning LPS administration, while the other 9 pigs survived beyond the experimental observation period of 3 h, although they were in a state of shock. No difference in LPS concentration was found between the survivors and the non-survivors. However, the serum TNF levels in non-survivors were significantly higher than in survivors when measured at 30 min after beginning LPS administration. In survivors, the peak increase in serum TNF levels was measured at 60 min after the beginning of LPS injection and returned rapidly to the baseline values. Although the role of TNF inducing rapid death seems to be dominant, the hemodynamic, hematology and blood chemistry disturbances seen during shock continued in survivors long after the return of TNF to baseline levels. These findings indicate that besides TNF other mediators are also involved in the LPS infusion-induced shock.

Key words: Endotoxin; Pig; Shock; TNF

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2. Introduction

The involvement of TNFα as a crucial mediator in shock, inflammation and cachexia has been proposed [1] on the basis of three different lines of evidence. First, increase in TNF levels was reported in animal models of shock and other forms of acute inflammation [2, 3]. The increase in TNF levels was correlated to the intensity of septic shock and was also found to occur in patients dying from meningococcal septicemia [4]. Second, antibodies against TNF protected septic shock in murine, rabbit and baboon models [5–7]. Third, administration of TNF itself can induce hemodynamic and laboratory changes, which are characteristics for septic shock [8, 9].

Some findings indicate, however, that differences exist between shock induced by LPS and shock induced by TNF itself. The kinetics of hemodynamic, hematologic and blood chemistry changes differ between the two kinds of shock [10]. Moreover, the plasma levels of TNF required to induce death are

much higher than the serum TNF levels induced by a lethal dose of LPS [3]. The treatment with antibody against TNF is not successful if given 1 h before administration of bacterial infusion [6]. These data indicate that besides TNF some other inflammatory mediators may play a role in the pathogenesis of endotoxic shock.

During our study on shock induced in pigs by infusion of LPS, we found an almost even distribution in the pig population of susceptibility to the lethal effects of LPS. Thus, this model provided an opportunity to determine the relationship between susceptibility to the lethal effects of LPS infusion and TNF levels, and the hemodynamic, hematologic and blood chemistry changes induced by LPS. The aim of the present work was to investigate the existence of such a relationship.

3. Materials and Methods

3.1. *Experimental set-up*

E. coli LPS (5 μg kg^{-1} of O111 B4, Serva) was infused into the superior mesenteric artery over 60 min in pigs (female, age 13–15 weeks) anesthetized with pentobarbital sodium (20 mg kg^{-1}, i.v.) after pre-medication with ketamine (20 mg kg^{-1}, i.m.). The animals were subsequently observed for a further period of 120 min. The surgical preparation was performed as described in detail elsewhere [11]. The core temperature was measured with a thermometer (Philips, HP 5311, Japan) attached to the liver. Mean arterial blood pressure (MABP) was continuously monitored by electromanometer using Statham P 23 dB strain gauge (Hato Rey, PR). Cardiac output (CO) was determined intermittently by thermodilution (WTI Computer, The Netherlands).

3.2. *Experimental protocols*

Group I (sham operated). Three animals were prepared as described above, except that instead of LPS, physiological saline was infused into the superior mesenteric artery. Blood (5 ml) was collected from pulmonary artery for laboratory measurements.

Group II (LPS-induced shock). Seventeen pigs were infused with 5 μg kg^{-1} of *E. coli* LPS into the superior mesenteric artery over a 60-min period and the animals were observed for an additional 120-min period. Blood (5 ml) was collected each time from aorta for LPS and TNF measurements at 10 min before, 10, 30 and 60 min after the start of LPS infusion, 60 and 120 min after termination of LPS infusion.

3.3. *Assay for plasma levels of LPS*

Blood for LPS assay was collected in plastic tubes (Falcon 2063, Oxnard, CA, U.S.A.) pre-filled with pyrogen-free heparin (Thromboliquine®, Organon, Oss, The Netherlands) at a final concentration of 30 U l^{-1}. After mixing, these tubes were immediately immersed in melting ice. Plasma was obtained by centrifugation at 160 × *g* at 4 °C for 10 min and plasma aliquots were stored at −70 °C. LPS was assayed with a chromogenic *Limulus* test [12]. All assays were performed in duplicate.

3.4. *Assay for serum levels of TNF*

Blood was collected in sterile tubes (Costar, Cambridge, MA). Serum was separated rapidly after coagulation by centrifugation and aliquots were stored at −20 °C until assay. TNF activity was determined by measuring the cytostatic effect of TNFα on the murine transformed fibroblast cell line L929 (a gift from W. Fiers, State University of Ghent, Belgium) [13, 14]. L929 cells were plated in a 96-well flat-bottomed microtiter plate (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well in 25 μl of RPMI-1640 culture medium. To quadruplicate wells, 25 μl medium (control), human recombinant TNF standard solutions (10, 100, 1000 U ml^{-1}) (Roche Research, Ghent, Belgium) or serum were added (final dilution 1:10). After incubation for 24 h in humidified atmosphere (7.5% CO_2) at 37 °C, 50 μl of a 10-μCi ml^{-1} [^{3}H]thymidine (Amersham Laboratories, Amersham, U.K.) solution was added. After 2 h of incubation L929 cells were harvested on glass fiber filtermats (Shatron Inc., Sterling, VA, U.S.A.). The uptake of [^{3}H]thymidine was measured by liquid scintillation spectroscopy. A standard curve of cytostasis by human recombinant TNF was obtained yielding progressive cytostasis ranging from 10–1000 U ml^{-1} of TNF. The bioactivity of TNF in experimental samples was deter-

mined in quadruplicate and compared against the standard curve.

3.5. *Determination of blood chemistry values*

Plasma glucose concentrations were measured by Glucoquant kit (Boehringer, Mannheim, F.R.G.). Laboratory values of blood hemoglobin, hematocrit as well as leukocyte and platelet counts were measured by hematology analyzers (Sysmex CC-108 and PI-100, Kobe, Japan, as appropriate).

3.6. *Statistical analysis*

All values are expressed as means ± SEM. The data were evaluated by the two-way analysis of variance (Friedman test) followed by a Wilcoxon-Wilcox test or two-tailed Mann-Whitney U test, as appropriate. A p value of 0.05 or less was considered statistically significant for all tests.

4. Results

4.1. *Sham-operated animals*

In sham-operated animals systemic hemodynamic variables and laboratory values were stable during the experiment (Table I, Group I).

4.2. *LPS-treated animals*

Eight of the 17 animals treated with LPS died within 30 min after LPS infusion was started (non-survivors), while the other 9 survived the experimental period of 3 h (2 h after termination of LPS infusion), though in a state of shock (survivors).

Systemic hemodynamics (Table I, Group II). In survivors, mean arterial blood pressure (MABP) gradually decreased and was significantly lower than the baseline from the end of LPS infusion period onwards. In contrast, MABP in the non-survivors dramatically dropped from 15–30 min after the start of LPS infusion. At 25–30 min MABP in this group

TABLE I

Effects of *E. coli* LPS infusion on hemodynamic and blood parameters in anesthetized pigs.

	MABP (mmHg)	CO (l min^{-1})	Temperature (°C)	Hb (mM l^{-1})	WBC (G l^{-1})	Platelet count	Blood glucose (mM l^{-1})
Group I: sham operated (n = 3)							
Control	99 ± 8	2.7 ± 0.4	38.6 ± 0.3	5.7 ± 0.4	13.5 ± 2.3	350 ± 49	6.0 ± 0.24
60 min	116 ± 8	2.8 ± 0.1	39.1 ± 0.5	6.0 ± 0.4	20.0 ± 5.0	340 ± 48	6.3 ± 0.26
120 min	114 ± 2	2.2 ± 0.1	39.2 ± 0.4	6.1 ± 0.5	20.0 ± 5.0	330 ± 49	5.8 ± 0.17
180 min	121 ± 8	2.4 ± 0.3	39.5 ± 0.4	6.3 ± 0.4	20.0 ± 6.0	330 ± 52	5.8 ± 0.36
Group II: LPS infusion-induced shock (n = 17)							
Survivors (n = 9)							
Control	108 ± 3	2.8 ± 0.3	39.3 ± 0.3	6.6 ± 0.2	11.5 ± 1.5	340 ± 41	5.3 ± 0.30
15 min	106 ± 5	2.1 ± 0.3	38.6 ± 0.2	7.0 ± 0.2	9.7 ± 1.2	330 ± 46	5.5 ± 0.30
30 min	89 ± 7	1.5 ± 0.1*	38.8 ± 0.3	7.5 ± 0.2*	5.4 ± 1.1*	260 ± 37*	5.6 ± 0.32
60 min	72 ± 7*	2.0 ± 0.2	39.3 ± 0.2*	7.5 ± 0.2*	4.5 ± 0.8*	230 ± 33*	5.0 ± 0.30
120 min	66 ± 6*	1.3 ± 0.1*	39.9 ± 0.3*	7.5 ± 0.2*	4.1 ± 1.2*	230 ± 38*	4.2 ± 0.20*
180 min	55 ± 5*	1.0 ± 0.1*	40.5 ± 0.3*	7.7 ± 0.3*	5.4 ± 1.1*	210 ± 32*	2.8 ± 0.40*
Non-survivors (n = 8)							
Control	110 ± 5	2.8 ± 0.1	39.3 ± 0.3	6.1 ± 0.3	11.4 ± 0.6	370 ± 34	5.4 ± 0.15
15 min	92 ± 8	1.9 ± 0.1	39.5 ± 0.4	6.5 ± 0.3	10.2 ± 0.8	320 ± 30	6.0 ± 0.33
30 min	28 ± 2*	1.0 ± 0.1*	39.9 ± 0.3*	6.7 ± 0.2*	6.1 ± 0.7*	260 ± 31*	9.4 ± 0.84*

Values are means ± SEM; n is number of observations. Abbreviations: MABP, mean arterial blood pressure; CO, cardiac output; Hb, hemoglobin; WBC, white blood cells. *, < 0.05 represents the probability values at different time periods compared to those at the baseline (control) calculated by the 2-way analysis of variance (Friedman test) followed by Wilcoxon-Wilcox' test.

was 28±2 mmHg and animals died 30 min after starting LPS infusion. In survivors there was a marked transient decrease in cardiac output from 25–30 min after starting LPS infusion. Subsequently, cardiac output returned to about baseline levels in spite of the continuous LPS infusion. From 120 min (1 h after stopping LPS administration) cardiac output again tended to decline, whereas the concentration of LPS in the circulation returned to the baseline levels. In non-survivors, cardiac output decreased quickly to one-third of baseline values within 25 min after the start of LPS infusion.

In non-survivors basal core temperature was higher by 1 °C than in survivors (Table 1, Group II, $p<0.05$, n=17). Temperature was elevated in both groups following LPS infusion.

Laboratory measurements (Table 1, Group II). LPS infusion induced a rapid increase in hemoglobin values, evident from 30 min after commencement of LPS infusion. This hemoconcentration was apparent toward the end of the observation period and did not improve after the LPS infusion was stopped. The LPS infusion was followed by a similar decrease in WBC and platelet counts at 30 min of LPS infusion in both survivors and non-survivors. After the LPS infusion was stopped, WBC and platelet counts in the survivors remained significantly lower than baseline. In non-survivors a clear hyperglycemia developed, while survivors showed severe hypoglycemia.

4.3. *LPS determinations (Fig. 1)*

The basal concentration of LPS was 520±47 ng l^{-1} (n=6) in aortic plasma prior to the start of LPS infusion. There were no differences in plasma LPS levels between survivors and non-survivors (460±40 ng l^{-1} and 580±78 ng l^{-1}, respectively, $n=3$ each). A peak in the LPS levels was reached at 30 min after the start of LPS infusion and remained constant till the end of the infusion period. The differences between peak concentrations observed in survivors and non-survivors were not statistically significant (920±190 ng l^{-1} and 1026±203 ng l^{-1}, n=3 each). The plasma LPS levels rapidly declined after the stoppage of LPS infusion and returned to the baseline in 60 min (560±162 ng l^{-1}, n=3).

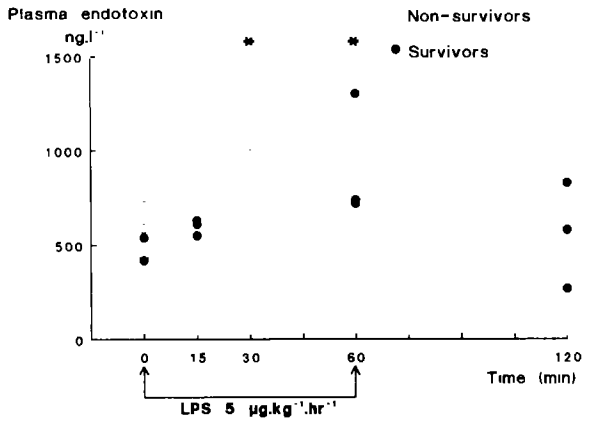

Fig. 1. Aortic plasma endotoxin concentrations (ng l^{-1}) after endotoxin (LPS) infusion into the superior mesenteric artery in anesthetized pigs. Endotoxin infusion was 5 µg kg^{-1} from 0–60 min. Values are means ± SEM. Closed circles indicate those animals that survived the observation period of 3 h after starting LPS infusion (survivors, n=3). Open circles indicate those animals that died in 30 min after starting LPS infusion (non-survivors, n=3). *, represents the probability values at different time periods compared to those at the baseline calculated by the 2-way analysis of variance (Friedman test) followed by Wilcoxon-Wilcox' test.

4.4. *TNF measurements (Fig. 2)*

Aortic blood samples processed as described contained a substance that was cytostatic for L929 mu-

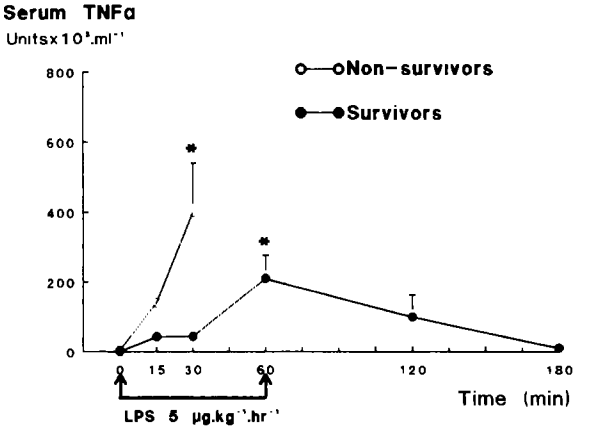

Fig. 2. Time course of the TNFα response to LPS infusion. Ordinate: TNFα levels in units × 10^3 ml^{-1} as determined by L929 bioassay in aortic serum. TNFα values in sham-operated group (n=3) remained under the detection limit (400 U ml^{-1}). Closed circles indicate the survivors (n=4), open circles indicate the non-survivors (n=5). Other details as in Fig. 1.

rine fibroblast line. In the survivors, TNF was either undetectable or just detectable in the serum until 30 min of LPS infusion; at 60 min TNF level was clearly increased, but thereafter it declined again. In contrast, a marked increase was detected in TNF release in non-survivors at the time of death.

5. Discussion

Continuous infusion of 5 μg kg^{-1} LPS caused death in 8 out of 17 pigs treated. Some possibilities might be put forward for explaining this split in the pig population, such as genetic control on the amount of TNF released following continuous LPS infusion and/or release of other mediators which might down-regulate the release of TNF. In this context, it is claimed that the release of PGE_2 might inhibit the release of TNF (quoted in review; ref. 15). In view of the fact that no difference was found in the plasma concentration of LPS between survivors and non-survivors, it seems unlikely that the difference in susceptibility to death is due to a difference in the clearance of LPS.

The death caused by LPS infusion seems to be closely related to the amount of TNF release induced by LPS. This finding is based on determinations of cytostasis against the target cell-line L929 (selectively sensitive to TNF; refs. 13 and 14) by serum obtained from LPS-treated pigs. It should be noted that the test sera used also contain a certain quantity of LPS and, possibly, interleukin 1 (IL-1) released either by a direct effect of LPS [16] and/or as a sequel to TNF release [16]. It has been mentioned that some other factors present in the serum (besides TNF), might affect activity against L929 cells [14]. However, circumstantial evidence is strongly in favor of the assumption that the cytostatic effect of serum from LPS-treated pigs is due to the TNF. This assumption is supported by our observations that LPS itself is not cytostatic against L929 cells (data not shown) and by findings of others that L929 cells are not susceptible to IL-1 [17]. Accordingly, it seems likely that death is caused in a certain percentage of LPS-infused pigs by a marked increase in TNF release as the main mediator for lethality.

The findings of different susceptibility to the lethal effect of LPS in a specified population of pigs provides an experimental model for determining the relation between lethal effect and endotoxic shock. In this respect, it is of interest that pigs surviving LPS infusion remained in a state of shock up to the end of the 120-min observation period, i.e., long after termination of LPS infusion and after the blood levels of LPS and of released TNF returned to basal values. The shock state was ascertained by systemic hypotension, low cardiac output, hemoconcentration, leukocytopenia and hypoglycemia. The results, indicating a shock state in surviving pigs long after LPS and TNF levels returned to baseline, suggest that other mediators besides or instead of TNF are responsible for the endotoxic shock observed. The release of these mediators (PAF, eicosanoids) might be evoked by LPS itself [16] and/or TNF.

In conclusion, we show here a clear distinction between LPS-induced death and LPS-induced shock. This finding implies that different mediators may be involved in these two events.

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