J Anesth (1994) 8:305–310

Journal of
Anesthesia

Therapy with antibody to tumor necrosis factor against endotoxin shock in rabbits

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Abstract: The purpose of this study was to determine the efficacy of treatment with anti-tumor necrosis factor (TNF) antibody in preventing the deleterious effects of endotoxin. Polyclonal anti-TNF antibody was produced by immunizing rabbits. Experiments were carried out on 16 rabbits intravenously infused with the lethal dose of lipopolysaccharide (LPS). Pretreatment with anti-TNF antibody resulted in protection from the development of hypotension and metabolic acidosis. The serum TNF level was significantly depressed in the antibody-pretreated group. Eighty-eight percent in the anti-TNF antibody-pretreated rabbits survived more than 48 h, whereas none of the rabbits who were given LPS alone survived over 24 h (LPS group). Prominent histopathological changes in the liver and kidney were evident in the LPS group. In contrast, pathologic changes in the tissue from the anti-TNF antibody group were considerably less prominent. These results support the idea that TNF plays a central role in mediating the pathophysiologic changes during endotoxin shock.

Key words: Shock, Antibody, Tumor necrosis factor, Endotoxin

Introduction

Septic shock continues to be associated with a high mortality rate despite the availability of a wide variety of treatment [1–4]. Tumor necrosis factor (TNF) has been implicated as a mediator of lethal endotoxemia [5,6]. Endotoxin is reported to be the most potent stimulus for the production of TNF [7,8]. Intravenous administration of endotoxin in human volunteers resulted in an increase in serum TNF level accompanied by fever, tachycardia, and systemic symptoms reaching a peak at 90 min following administration [9]. The infusion of TNF resulted in hemodynamic collapse, metabolic acidosis, and tissue injury resembling septic shock [5,6]. It has been suggested that the TNF released from macrophages may account for many of the acute, insufferable consequences of endotoxin shock. The purpose of this study was to determine the efficacy of treatment with an anti-TNF antibody in preventing the deleterious effects of endotoxin in rabbits.

Address correspondence to: K. Ishizaki
Received for publication on November 24, 1992; accepted on December 16, 1993

Materials and methods

Antibody production

Polyclonal anti-TNF was produced by immunizing rabbits with human recombinant TNF (5×10^6 units·ml^{-1}, <20 ng endotoxin·mg^{-1}) which was provided by Asahi Chemical Industry (Nobeoka, Japan). The initial immunization was made with 5×10^5 units TNF i.m. Booster intramuscular injections given on weeks 2, 4, 6, 8, and 10 consisted of 10^6 units TNF. Eleven weeks later, blood was collected in plastic ware, centrifuged and the serum was frozen with 0.05% NaN_3.

Neutralizing capacity of the antibody

The neutralizing capacity of the antibody was determined by the TNF-specific enzyme-linked immunosorbent assay (ELISA) method described previously [10]. In short, rabbit serum that was diluted from 1 to 10^6 was added to 96-well immunoassay plates coated with 2 units·well^{-1} of the human recombinant TNF. Before adding rabbit serum, the plates were blocked with 1% bovine serum in phosphate buffered saline for 1 h at room temperature to prevent nonspecific binding. A standard titration curve was obtained by making serial dilutions of a known sample of anti-TNF_α antibody

(Genzyme, Boston, MA). The amount of anti-TNF antibody bound to the wells was quantified by sequential incubation with peroxidase-conjugated antibody to the human recombinant TNF antigen-antibody complex (Asahi Chemical Industry). The color reaction was stopped with 1.0 MH_2SO_4, after which extinction at 495 nm was measured with a microELISA autoreader (3350UV, Bio-Rad, New York, NY). The lower detection limit of the ELISA was 0.1 units·ml^{-1}.

Animal preparation

Japanese white rabbits, male, weighing 3 kg were utilized. Rabbits were fasted overnight. The rabbits were anesthetized with a single dose of ketamine i.m. (30 mg·kg^{-1}), and were restrained in the supine position; the femoral venous and arterial catheters were then inserted surgically using a local anesthetic (2 ml, 1% mepivacaine). The rectal temperature was monitored and maintained at a physiological level throughout the experiment by a warming blanket. After recovery from the anesthesia, the rabbits were restrained with a stereotaxic apparatus (Clea Products, CL-4521, Tokyo).

Physiologic monitoring

Mean arterial pressure (MAP) was measured using fluid-filled catheters and transducers, and recorded with a polygraph (RMC-1200, Nihon Koden, Tokyo, Japan). An electronic thermometer was attached to obtain the temperature. Arterial blood gases were determined on an analyzer (ABL2, Radiometer, Copenhagen, Denmark). Blood samples were collected to measure leukocyte counts and β-glucuronidase. β-glucuronidase was measured as a lysosomal enzyme according to the method of Fishmann.

Experimental design

Sixteen rabbits were divided into two groups, the anti-TNF antibody group and the LPS group. All animals received intravenous lactated Ringer's solution 10 ml·kg^{-1}. After baseline parameters were established, 4 ml of rabbit serum with or without anti-TNF antibody was infused at 30–45 min before intravenous injection of lipopolysaccharide (LPS, Difco Laboratories, Detroit, MI 67 μg·kg^{-1}) Physiological variables were monitored until 5 h after the injection of LPS. Two ml of blood was obtained for assay of TNF level at 30-min intervals. In the course of the experiment, animals recognized as moribund (in general, MAP < 30 mmHg) were killed by intravenous injection of sodium pentobarbital. Necropsy was performed, and tissues were prepared for histologic examination.

TNF assays

TNF assay was quantified by measuring the cytotoxic effect of rabbit serum on L929 cells using methods described previously [11]. Briefly, fibroblast cultures (L929, 2×10^4 cells) were incubated with serial dilutions of the test sample in 96-well plates in the presence of actinomycin D. The supernatants were removed, and cell lysis was determined by staining the plates with crystal violet (Sigma, St. Louis, MO). Dye uptake was quantified by using a microELISA autoreader. The titer of TNF is expressed in units·ml^{-1} and was chosen as the reciprocal of the dilution necessary to cause 50% cell destruction. The presence of TNF was confirmed by the use of a TNF antiserum (Genzyme), which neutralized the lytic activity of sera and macrophage supernatant.

Statistical Analysis

Data were analyzed using Student's *t*-test to determine significant differences. Fisher's exact test was used to determine significant differences between groups with respect to survival rates. The differences were considered significant when $P < 0.05$.

Results

In vitro neutralization of anti-TNF antibody activity is shown in Fig. 1. Dilution to 10^5 revealed the anti-TNF activity. MAP, heart rate, blood gas analysis, and β-glucuronidase are shown in Table 1. In the LPS group, MAP fell 15%–20% over 100–500 min. In contrast, MAP remained in the normal range in rabbits pretreated with the antibody (Fig. 2). The leukocyte count decreased in both groups, but the decrease was greater in the LPS group than in the anti-TNF antibody group (Fig. 3). The β-glucuronidase levels were increased at 2 h after LPS injection in the LPS group, but were not increased in the anti-TNF antibody group (Fig. 4). Blood gas analysis revealed metabolic acidosis in the LPS group (Fig. 5). The serum TNF level was increased at 30 min after LPS administration, peaked at 90 min, and returned to near the baseline level by 180 min. The mean TNF level was increased significantly in the LPS group, when compared with the anti-TNF antibody group (Fig. 6). As shown in Table 2, 86% of the anti-TNF-treated rabbits survived over 24 h, whereas none of the animals in the LPS group did so. Light microscopy findings, which are summarized in Table 3, include fibrin microthrombus and vacuolytic changes in the liver and fibrin deposits in the kidneys of the LPS group. In contrast, pathologic changes in the tissue from the anti-TNF antibody group were considerably less prominent.

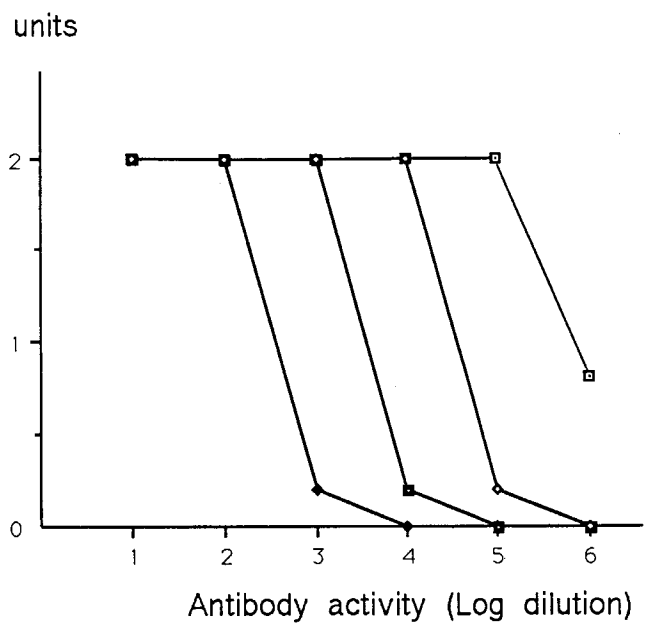

Fig. 1. In vitro neutralization of tumor necrosis factor (TNF) activity measured by enzyme-linked immunosorbent assay (ELISA). Each well was coated with 2 units of human recombinant TNF, and was mixed with various diluted antibody. *Open squares*, Rabbit serum; *open diamonds*, 20 000 units of antibody; *closed squares*, 2000 units of antibody; *closed diamonds*, 200 units of antibody

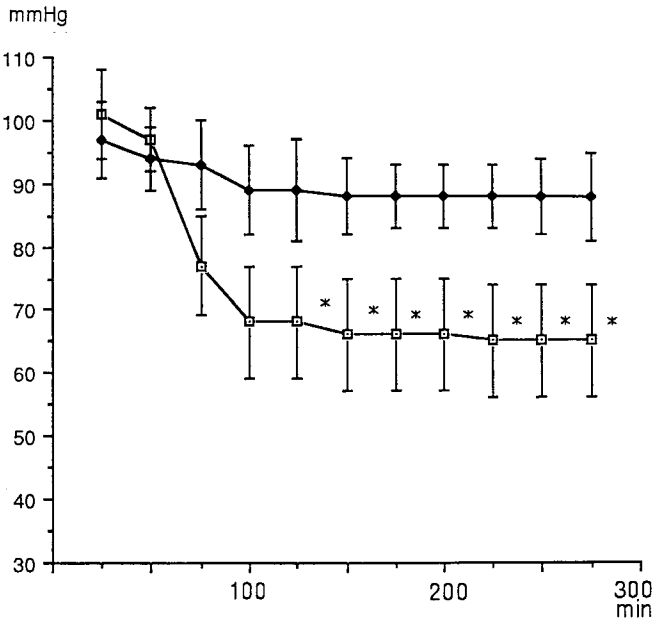

Fig. 2. Mean arterial pressure changes following intravenous injection of lipopolysaccharide (LPS) with or without anti-TNF antibody. Data is presented as mean ± SEM. * $P < 0.05$ versus LPS group. *Closed diamonds*, anti-TNF group; *open squares*, LPS group

Table 1. Mean arterial pressure (MAP), heart rate, blood gas analysis, and β-glucuronidase in two groups at varying times

	LPS group			Anti-TNF antibody group		
Time (min)	0	120	240	0	120	240
MAP (mmHg)	102 ± 7	68 ± 9[c]	64 ± 9[c]	97 ± 6	89 ± 7[a]	88 ± 5[a]
Heart rate (beats·min^{-1})	230 ± 29	267 ± 36	280 ± 37	240 ± 34	257 ± 39	259 ± 42
pH	7.45 ± 0.4	7.33 ± 0.5[c]	7.30 ± 0.6[c]	7.45 ± 0.4	7.44 ± 0.5[a]	7.44 ± 0.4[a]
Pao_2 (mmHg)	94.9 ± 11.9	96.5 ± 10.5	99.6 ± 11.4	93.4 ± 10.4	94.0 ± 9.8	94.5 ± 9.9
$Paco_2$ (mmHg)	32.0 ± 4.2	24.6 ± 4.9[c]	23.6 ± 5.2[c]	32.1 ± 3.9	27.5 ± 4.4	26.4 ± 4.6
BE (mEq·L^{-1})	−1.6 ± 0.4	−12.2 ± 2.1[e]	−14.1 ± 3.5[e]	−1.8 ± 0.4	−4.2 ± 1.2[ac]	−5.8 ± 1.1[ad]
β-glucuronidase (units·ml^{-1})	428 ± 43	1446 ± 156[e]	2886 ± 227[e]	440 ± 52	744 ± 69[ac]	754 ± 87[bc]

Mean ± SEM.
[a] $P < 0.05$ versus LPS group at the same time.
[b] $P < 0.01$ versus LPS group at the same time.
[c] $P < 0.05$ versus baseline within each group.
[d] $P < 0.01$ versus baseline within each group.
[e] $P < 0.001$ versus baseline within each group.
BE, base excess.

Table 2. Survival rate and time following intravenous injection of LPS with or without anti-TNF antibody. Mean ± SEM

	LPS group	Anti-TNF antibody
Survival at 24 h	1/8	7/8*
Mean survival time	12.5 ± 4.7	48H<*

* $P < 0.05$ between groups.

Table 3. Histopathologic changes in rabbits

	LPS group	Anti-TNF antibody
Liver		
Focal necrosis	7/8	1/8
Sinusoidal fibrin deposit	6/8	1/8
Kidney		
Glomerular fibrin deposit	6/8	1/8

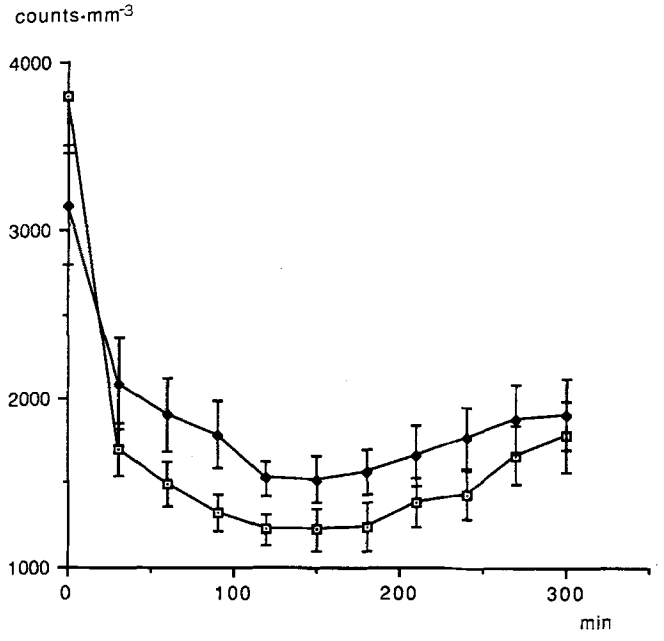

Fig. 3. Leukocyte count changes following intravenous injection of LPS with or without anti-TNF antibody. Data is presented as mean ± SEM. *Closed diamond*, anti-TNF group; *open square*, LPS group

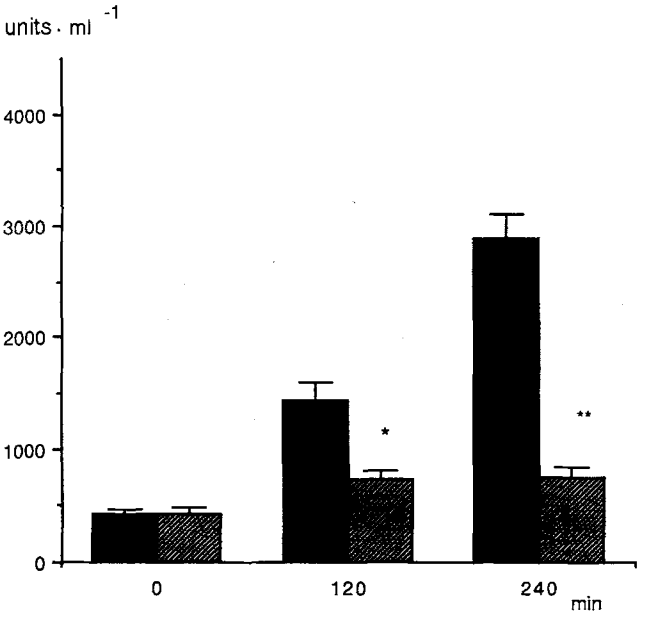

Fig. 4. Increase in β-glucuronidase following intravenous injection of LPS with or without anti-TNF antibody. $*P < 0.05$; $**P < 0.01$ between groups. *Hatched bars*, anti-TNF group; *closed bars*, LPS group

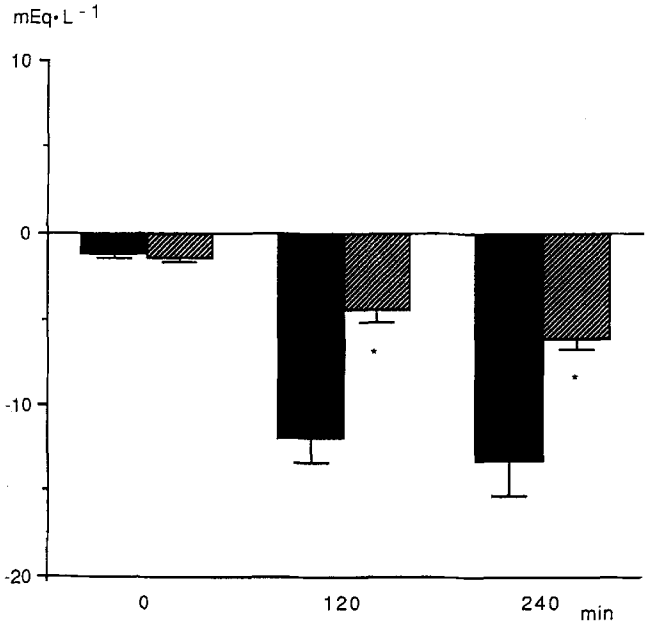

Fig. 5. Increase in base excess following intravenous injection of LPS with or without anti-TNF antibody. Data is presented as mean ± SEM. $*P < 0.05$ between groups. *Hatched bars*, anti-TNF group; *closed bars*, LPS group

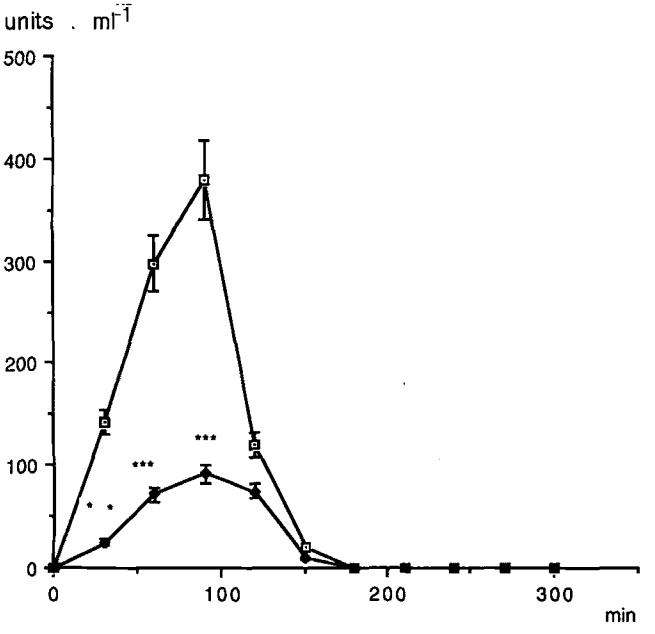

Fig. 6. Rabbit serum TNF activity measured by cytotoxic on L929 cells following intravenous injection of LPS with or without anti-TNF antibody. Data is presented as mean ± SEM. $**P < 0.01$, $***P < 0.001$ between groups. *Closed diamonds*, anti-TNF group; *open squares*, LPS group

Discussion

We examined the efficacy of an anti-TNF antibody against lethal LPS injection. Anti-TNF antibody activity was determined using the ELISA method. Dilution of antibody serum to 10 revealed anti-TNF activity. Four milliliters of antibody serum was diluted 50-fold when injected in the animals (body weight: 3 kg) and 1000 units of serum TNF was neutralized by 4 ml of the antibody serum. Our findings revealed that the dose of the antibody serum given was sufficient to neutralize the serum TNF released by LPS. Human TNF and rabbit TNF have a similar structure and are cross-reactive [12]. Our data indicated that the anti-TNF antibody bound to serum TNF in the rabbits and that the serum TNF activity was subsequently decreased. As there was no method available for accurately measuring rabbit TNF, we measured the serum TNF level by the L929 cell method [11]. Serum TNF levels has been reported to be increased from 60 to 180 min in healthy volunteers given endotoxin [9]. Serum TNF showed a similar response in the LPS group of our experiment, but the increase in TNF activity was prevented in the anti-TNF antibody group.

The anti-TNF antibody also prevented hypotension, metabolic acidosis, and the release of lysosomal enzymes. Ulevitch et al. [13] reported that the lethal dose of LPS in the rabbit is 50 $\mu g \cdot kg^{-1}$ and that the hypotensive response is characterized by a decrease in MAP after injection of LPS followed by a further decline in MAP. Intravenous injection of LPS induced hypotension and low cardiac output [14]. Hypotension and inadequate tissue oxygenation resulted in the destruction of cells. Survival of rabbits receiving the anti-TNF antibody may be explained by improved peripheral perfusion in that their MAP was higher than controls and their cardiac output may have been better [15].

Other factors also may contribute to changes in the survival rate of animals receiving the anti-TNF antibody. TNF induces the synthesis of monokines, such as interleukin-1 β (IL-1 β) and interleukin-6 (IL-6), which have been implicated in the pathophysiology of infection [16,17]. Passive immunization against TNF attenuated the appearance of IL-1 β and IL-6, indicating that TNF was the initial stimulus for the release of these cytokines during the septic syndrome [18]. Furthermore administration of recombinant IL-1 or IL-6 induced characteristic physiologic derangements associated with injury, including fever [19]. Okusawa et al. [20] described a shock-like state in rabbits after infusion of a high dose of IL-1, and Waage et al. [21] reported that IL-1 could potentiate the lethal effects of TNF. Endo et al. [22] suggested that endotoxin and extremely high levels of TNF, or simultaneous elevation of IL-1 β and IL-6, were related to the onset of septic shock in critical patients.

Other worker have demonstrated synergism between TNF and LPS [23]. For example, when LPS was added to an infusion of TNF, there was a marked increase in organ damage and a decrease in the neutrophil count [23]. Our study also suggested that there was a synergistic interaction between LPS and TNF. Prominent pathologic changes were observed after injection of LPS and the anti-TNF antibody had a protective effect on the liver and kidney. Antibody pretreatment resulted in the prevention of focal necrosis and a fibrin deposit.

A number of studies have provided evidence that TNF produces changes between neutrophil and endothelial cells [24,25]. Leukopenia due to LPS is a well-known phenomenon. TNF is a product of macrophages activated by LPS and also causes a decrease in the neutrophil count [24]. Binding of TNF to neutrophils results in enhanced adherence of neutrophil to endothelial cells. Remick et al. [25] reported cytoplasmic blebbing and gap formation between endothelial cells after injection of TNF.

Macrophages produce LPS and TNF in response to prostaglandins, in particular Prostaglandin E_2 (PGE_2) [26,27]. Kettelhut et al. [28] provided evidence that cyclooxygenase inhibitors provided protection from rapid killing, eventually lethality and other pathophysiologic changes after intravenous injection of recombinant TNF. Cyclooxygenase inhibitors, which prevent the production of prostanoids and vasodilators, improve hemodynamic stability [29].

Another mechanism for LPS-induced pathophysiological changes is considered. Hypotensive changes were mediated in part by endothelium-derived relaxing factors induced by endothelial cells, either directly by LPS or indirectly by TNF [30].

Debets et al. [31] has demonstrated that sepsis accompanied by detectable plasma TNF levels has a high mortality. The concept of a primary role of TNF in sepsis is supported by a positive correlation between elevated TNF levels and mortality in patients [21,22,32].

The present study provides support for the concept that TNF is an essential mediator of LPS-induced injury and initiates the primary changes which lead to the cascade of events culminating in lethal hypotensive shock.

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