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COMBINATION IMMUNOTHERAPY WHICH NEUTRALISES THE EFFECTS OF TNFα AND IL-1β ATTENUATES THE CIRCULATORY FAILURE AND MULTIPLE ORGAN DYSFUNCTION CAUSED BY ENDOTOXIN IN THE RAT

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Pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) or interleukin-1β (IL-1β) are implicated in the pathogenesis of septic shock. Here we investigate the role of endogenous TNFα and IL-1β on (i) the circulatory failure, (ii) the multiple organ dysfunction syndrome (MODS) and (iii) the induction of the inducible isoform of nitric oxide (NO) synthase (iNOS) caused by endotoxin (LPS) in the anaesthetised rat. Here we demonstrate that (i) a polyclonal antibody against TNFα, (ii) a polyclonal antibody against IL-1, (iii) co-administration of polyclonal antibodies against TNFα and IL-1 and (iv) neutralisation of the effects of both TNFα and IL-1 with one polyclonal antibody directed against both cytokines reduces the circulatory failure, the liver injury/dysfunction, the pancreatic injury (but not the renal dysfunction) caused by endotoxin in the rat. The beneficial effects of these interventions on haemodynamics and organ injury/dysfunction are most likely due to prevention of the induction of iNOS. The two different interventions which neutralised the effects of both TNFα and IL-1 were superior in reducing the circulatory failure and the organ injury caused by endotoxin in the rat, than single interventions aimed at neutralising the effects of either cytokine. Thus, we propose that interventions which are able to neutralise the effects of both TNFα and IL-1 (combination immunotherapy) may be of benefit in the treatment of patients with septic shock.

Key words: *cytokines, combination immunotherapy, tumour necrosis factor, interleukin-1, multiple organ dysfunction, multiple organ failure, nitric oxide synthase, septic shock, endotoxemia*

INTRODUCTION

The pro-inflammatory cytokines tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β) have been implicated in the pathophysiology of many cardiovascular disorders (1—3) including circulatory shock (4—9). Administration of TNFα or IL-1β alone or in combination with low doses of endotoxin mimics several features of the pathophysiology of circulatory shock including hypotension and organ injury (4—6). Pronounced rises in the serum levels of TNFα and IL-1β occur in experimental endotoxaemia (4—12). More

importantly, enhanced serum concentrations of TNFα and IL-1β have been documented in human subjects with sepsis and septic shock (13, 14). In addition, antibodies directed against TNFα or IL-1β exert protective effects in various animal models of endotoxin shock (15, 16).

Nitric oxide (NO) is a potent endogenous vasodilator autacoid produced by NO synthase (NOS) in many mammalian cells. Activation of macrophages and other cells with endotoxin or pro-inflammatory cytokines such as TNFα or IL-1β leads to the expression of an 'inducible' isoform of NOS (iNOS) (17—19). An enhanced formation of NO following the induction of iNOS has been implicated in the pathogenesis in a number of disease including circulatory shock of various aetiologies. For instance, an overproduction of NO contributes importantly to the severe, therapy-refractory hypotension and vascular hyporeactivity ('vasoplegia') to vasoconstrictor agents in animal models of endotoxic and haemorrhagic shock (20, 21).

The definition of shock does not include the presence of a multiple organ dysfunction syndrome (MODS), which is defined as the presence of altered organ function in acutely ill patients, such that homeostasis cannot be maintained without intervention. Primary MODS is a direct result of a well-defined insult in which organ dysfunction occurs early and is due directly to the specific insult. In contrast, secondary MODS develops as a consequence of the host response and is identified within the context of the systemic inflammatory response syndrome (SIRS). The progression of shock or SIRS to MODS is associated with an increase in mortality from 25-30% (in the absence of MODS) to 90—100% (22, 23).

Here we investigate the role of endogenous TNFα and IL-1β in the pathophysiology of the circulatory failure and MODS caused by LPS in the rat. Thus, we have elucidated the effects of the pre-treatment of rats (prior to the administration of LPS) with (a) a polyclonal antibody against TNFα, (b) a polyclonal antibody against or IL-1β, (c) a single polyclonal antibody which neutralises the effects of TNFα and IL-1β or (d) with a co-administration of two polyclonal antibodies which neutralise the effects of TNFα and IL-1β, respectively on (i) the circulatory failure, (ii) the MODS (e.g. liver dysfunction and injury, renal dysfunction) and (iii) the induction of iNOS caused by endotoxin (LPS) in the anaesthetised rat.

MATERIALS AND METHODS

Cell culture

Rat aortic smooth muscle cells (RASM) were cultured in RPMI medium, supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum (21). Cells were cultured in 96-well plates with 200%l culture medium until they reached confluence. To induce iNOS in RASM, fresh culture

medium containing tumour necrosis factor-α (TNFα; 10 U·ml^{-1}), interleukin-1β (IL-1β; 10 U·ml^{-1}) or TNFα+IL-1β (both 10 U·ml^{-1}) were added to the cells. Nitrite accumulation in the cell culture medium was measured after 24 h. To assess the effects of sheep polyclonal antibodies against human TNFα, IL-1β or a mixture of PAb against TNFα and IL-1β on nitrite production, PAb were added to the cells 30 min prior to the cytokines.

Measurement of nitrite production

The amount of nitrite, an indicator of NO synthesis, in the supernatant of RASM was measured by the Griess reaction (24) by adding 100 ml of Griess reagent to 100 ml samples of unfiltered serum or supernatant. The optical density at 550 nm (OD_{550}) was measured using a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculated by comparison with OD_{550} of standard solution of sodium nitrite prepared in control serum or culture medium.

Measurement of haemodynamic changes

Male Wistar rats (240—320 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetised with thiopentone sodium (Trapanal^{R}; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Statham, Oxnard, CA, U.S.A.) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, U.S.A.). The femoral vein and jugular vein were cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 15 min. After recording baseline haemodynamic parameters animals received vehicle for LPS (1 ml kg^{-1} i.v., saline, $n = 5$) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1} i.v. in 0.3 ml of saline, $n = 38$) as a slow injection over 10 min. At 6h after LPS, blood was taken to measure the changes in the serum levels of various biochemical marker enzymes of MODS (see below). Animals were divided into 8 groups which received injections of: *(1)* vehicle (saline, 1 ml kg^{-1} i.v.) plus vehicle for LPS (saline), $n = 4$; *(2)* vehicle (saline, 1 ml kg^{-1} i.p.) plus LPS, $n = 12$; *(3)* an ovine, polyclonal antibody against human TNFα (30 mg·kg^{-1} i.v.) plus LPS, $n = 6$; *(4)* an ovine, polyclonal antibody against human against human TNFα (100 mg·kg^{-1} i.v.) plus LPS, $n = 6$; *(5)* an ovine, polyclonal antibody against human IL-1β (30 mg·kg^{-1} i.v.) plus LPS, $n = 6$; *(6)* an ovine, polyclonal antibody against human IL-1β (100 mg·kg^{-1} i.v.) plus LPS, $n = 6$; *(7)* a co-administration of two polyclonal antibodies against human TNFα and IL-1β (both 30 mg·kg^{-1} i.v.), $n = 7$; and *(8)* an ovine, polyclonal antibody which neutralises the effects of both human TNFα and IL-1β (100 mg·kg^{-1} i.v., 30 min prior to LPS), $n = 6$. All of the antibodies were administered at 30 min prior to injection of LPS.

Quantification of liver, renal, or pancreatic injury

At 6 h after the injection of LPS, 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from a catheter placed in the carotid artery. The blood sample was centrifuged (6,000 rpm for 3 min) to prepare serum. All serum samples were analysed within 24 h by a contract laboratory for veterinary, clinical chemistry (Vetlab Services, Sussex, U.K.). The following marker enzymes were measured in the serum as biochemical indicators of multiple organ failure syndrome (MODS): Liver dysfunction and failure were assessed by measuring the rises in serum levels of

alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury); aspartate aminotransferase (AST, a non-specific marker for hepatic parenchymal injury) and bilirubin (an indicator of liver dysfunction/failure) (21). Renal dysfunction and failure were assessed by measuring the rises in serum levels of creatinine (an indicator of reduced glomerular filtration rate, and hence, renal failure) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism). The increase in the serum levels of lipase served as an indicator of pancreatic injuries.

Nitric oxide synthase assay

NOS activity was measured as the ability of tissue homogenates to convert L-[^{3}H]arginine to L-[^{3}H]citrulline (21). Lungs and livers were removed at 6 h after LPS and frozen in liquid nitrogen. Frozen organs were homogenised on ice with an Ultra-Turrax T 25 homogenizer (Janke Kunkel, IKA Labortechnik, Staufen i. Br., Germany) in a buffer composed of: Tris-HCl 50 mM, EDTA 0.1 mM, EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethylsulphonyl fluoride 1 mM (pH 7.4). Briefly, tissue homogenates (30 ml, approx. 60 mg protein) were incubated in the presence of [^{3}H]-L-arginine (10 mM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 mM) and calcium (2 mM) for 25 min at 25C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, U.S.A.). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-citrulline formation independent of a specific NOS activity. Experiments in the presence of NADPH, without calcium and with 5 mM EGTA, measured the calcium-independent iNOS activity, which was taken to represent iNOS activity. Protein concentration was measured spectrophotometrically in 96-well plates with Bradford reagent (25), using bovine serum albumin as standard.

Measurement of the serum concentrations of TNFα and interferon-(IFNγ)

The content of TNFα (at 90 min after LPS) and IFNγ (at 6 h after LPS) in serum samples (50µl) were determined by ELISA (Mouse TNFα ELISA kit and Mouse IFNγ ELISA kit, Genzyme, Cambridge, MA, U.S.A.) in 96-well plates according to the manufactures protocol. The absorbency of each well was measured at 450 nm using a Molecular microplate reader (Anthos Labtec Instruments, Richmond, CA, U.S.A.).

Materials

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), NADPH, noradrenaline bitartrate, MOPS, H_2O_2, Na_2HPO_4, foetal calf serum, L-glutamine, tetramethylbenzidine, NaH_2HPO_4, HTAB, N^G-methyl-L-arginine, Tris-HCL, EDTA, EGTA, 2-mercaptophanol, acetic acid, phenylmethylsulphonyl fluoride, HEPES buffer, Bradford reagent, bovine serum albumin and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Buckinghamshire, U.K.). Tetrahydrobiopterin (6R-L-erythro-5,6,7,8-tetrahydrobiopterin) was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). TNFα and IL-1β were human recombinant forms from RD (Abingdon, Oxon, U.K.). The sheep polyclonal antibodies (PAb) against human TNFα, IL-1β or the combination of PAb against human TNFα and IL-1β were a generous gift from Dr D. Smith (Therapeutic Antibodies Ltd., London, U.K.).

Statistical analysis

A one-way analysis of variance (ANOVA) followed by, if appropriate, a Dunnetts *post hoc* test was used to compare means between groups. A P value of less than 0.05 was taken as significant.

RESULTS

Effects of polyclonal antibodies against TNFα and/or IL-1β on the increase in nitrite formation caused by cytokines in cultured rat aortic smooth muscle cells (RASM)

Activation of RASM with either TNFα, IL-1β or TNFα and IL-1β resulted within 24 h in a significant increase in nitrite in the cell supernatant from 1.5 ± 0.2 μM (control) to 9.2 ± 1.1 μM, 16.1 ± 1.9 μM or 22.3 ± 3.2 μM, respectively ($P > 0.05$, $n = 9$, *Fig. 1a, b, c*). Pretreatment of activated RASM with polyclonal antibodies against TNFα, IL-1β or a combination of polyclonal antibodies against TNFα and IL-1β resulted in a concentration-dependent inhibition of the nitrite formation (*Fig. 1a, b, c*). In addition, we tested whether there is a cross-reactivity between the polyclonal antibodies and the non-directed cytokine (e.g. TNFα and polyclonal antibody against IL-1β, IL-1β and polyclonal antibody against TNFα). Therefore, the production of nitrite in RASM were induced by (i) TNFα in the presence or absence of a polyclonal antibody against IL-1β, (ii) IL-1β in the presence or absence of a polyclonal antibody against TNFα, or (iii) TNFα and IL-1β in the presence or absence of polyclonal antibodies against either TNFα or IL-1β (*Fig. 1d*). However, the polyclonal antibody against TNFα had no effect on the increase in the formation of nitrite caused by IL-1β and the polyclonal antibody against IL-1β had no effect on the increase in the formation of nitrite caused by TNFα. In addition, incubation of RASM activated with TNFα and IL-1β in the presence or absence of a polyclonal antibody against either IL-1β or TNFα only resulted in a partial inhibition of the increase in the formation of nitrite (*Fig. 1d*). Thus, we did not observe any cross-reactivity between the antibodies and the non-directed cytokine.

Effects of polyclonal antibodies against TNFα and/or IL-1β on the circulatory failure caused by endotoxin in vivo

Baseline values for MAP and HR of all of the animal groups studied ranged from 114 ± 5 to 119 ± 4 mmHg, and from 389 ± 18 to 408 ± 21 beats $\cdot$ min^{-1}, and were not significantly different between groups ($p < 0.05$). Administration of LPS (10 mg $\cdot$ kg^{-1} i.v.) caused (within 15 min) a fall in MAP from 118 ± 5 to 66 ± 6 mmHg ($n = 12$, $P > 0.05$). This fall in MAP, however, was transient so that the mean MAP values of rats subjected to injection of LPS were in the range of 95 to 105 mmHg from 60 to 180 min after injection of LPS. This was

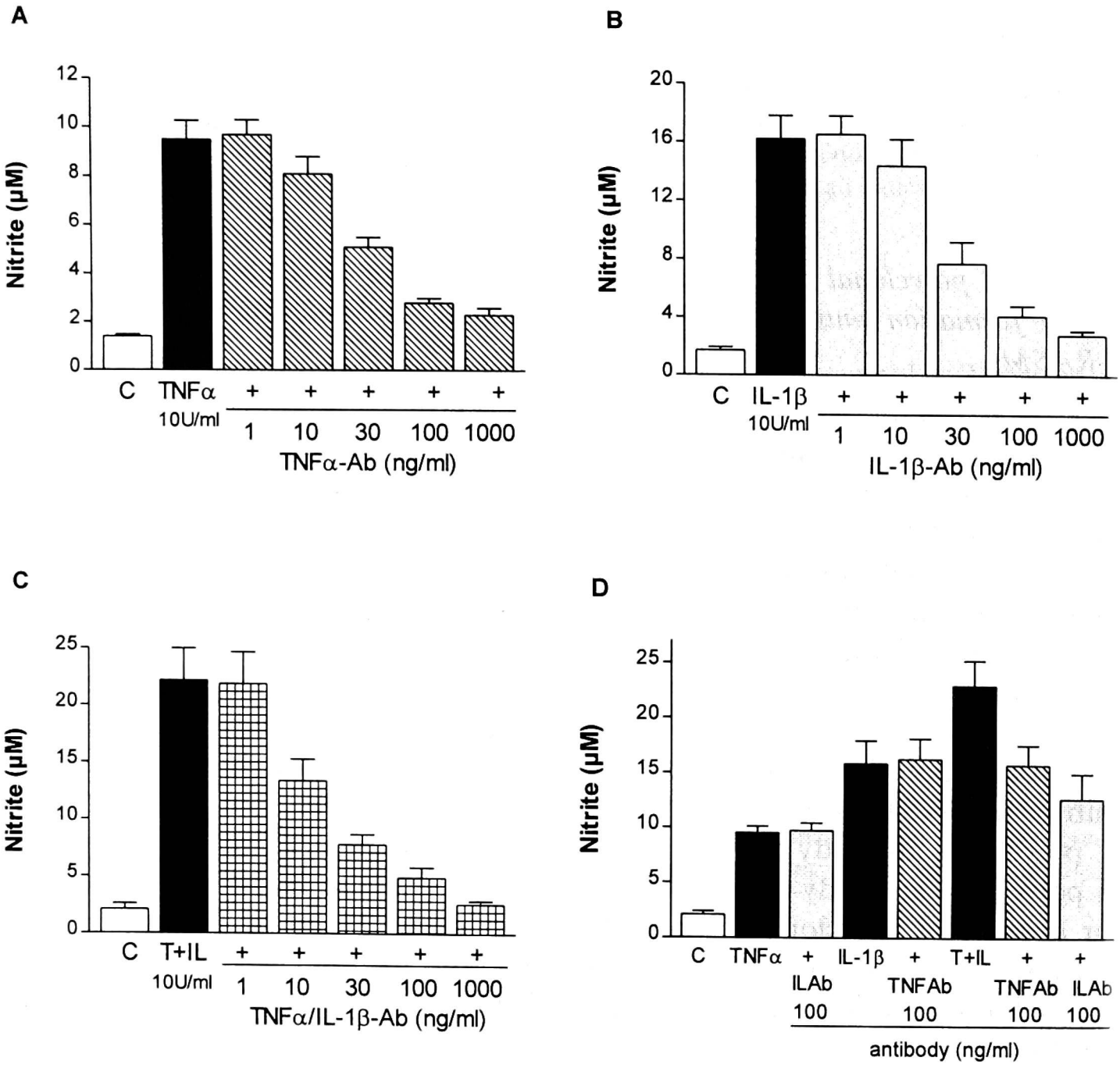

Fig. 1. Effects of (A) a polyclonal antibodies against TNFα (TNFα-Ab), (B) a polyclonal antibody against IL-1β (IL-1β-Ab) or (C) a single polyclonal antibody which neutralises the effects of both TNFα and IL-1β (TNFα/IL-1β-Ab) on the accumulation of nitrite in the supernatant of RASM activated with TNFα (10 U/ml), IL-1β (10 U/ml) or TNFα and IL-1β (T+IL; both 10 U/ml), respectively. (D) RASM were activated with either TNFα (10 U/ml), IL-1β (10 U/ml) or TNFα and IL-1β (T+IL; both 10 U/ml) in the presence or absence of a polyclonal antibody against IL-1β (ILAb; 100 ng/ml) or TNFα (TNFAb; 100 ng/ml). Please note that Panel D demonstrates that their is no cross-reactivity between the specific polyclonal antibody used and the non-directed cytokine (e.g. TNFα and IL-1β-Ab; IL-1β and TNFα-Ab). The above antobodies were added 30 min prior to immunostimulants. Data are expressed as means ± s.e.mean of $n = 9$ observations from three separate experiments.

followed a progressive decline in MAP, so that the MAP of LPS-rats was 81 ± 5 mmHg at 360 min (*Fig. 2*). Pretreatment of LPS-rats with (i) the high dose of the polyclonal antibody against TNFα (100 mg · kg^{-1}, i.v.), (ii) the high dose of the polyclonal antibody against IL-1β (100 mg · kg^{-1}, i.v.), (iii)

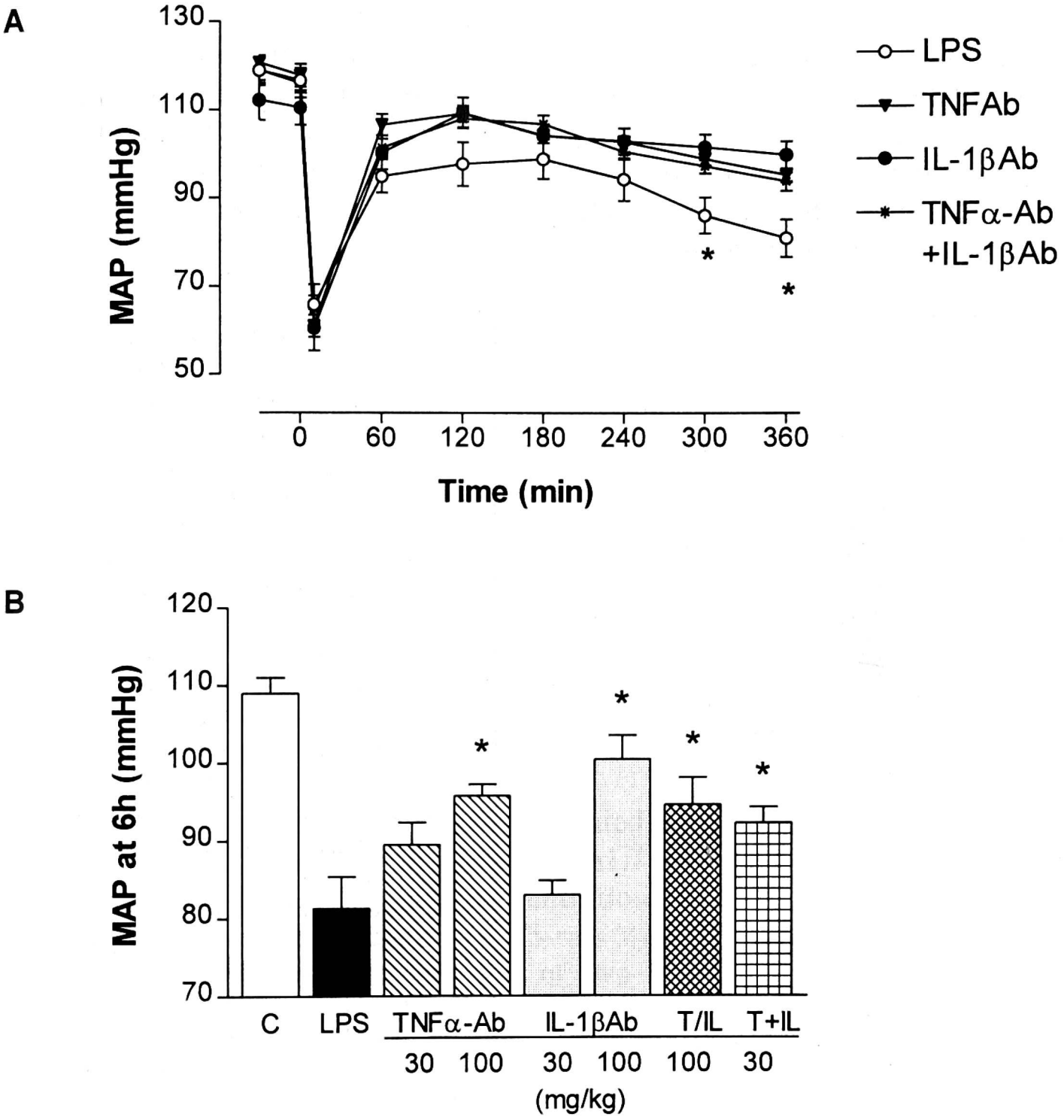

Fig. 2. Effects of (i) a polyclonal antibody against TNFα, (ii) a polyclonal antibody against IL-1, (iii) co-administration of polyclonal antibodies against TNFα and IL-1 and (iv) neutralisation of the effects of both TNFα and IL-1 with one polyclonal antibody directed against both cytokines on the delayed circulatory failure caused by endotoxin in the anaesthetised rat. Depicted are the changes in mean arterial blood pressure (MAP) (a) over 6 h and (b) at 6 h after treatment with *E. coli* lipopolysaccharide (LPS; 10 mg·kg^{-1}, i.v. at time 0; $n = 6$—12 per group). Different groups of LPS-rats received injection of vehicle (saline, 0.6 ml kg^{-1} h^{-1}, open circle or solid column, $n = 12$), a polyclonal antibody against TNFα (TNFα-Ab; 30 or 100 mg·kg^{-1} i.v., closed triangles (100 mg·kg^{-1}) or hatched columns, $n = 6$ per group), a polyclonal antibody against IL-1β (IL-1βAb; 30 or 100 mg·kg^{-1} i.v., closed circles (100 mg·kg^{-1}) or stippled columns, $n = 6$ per group), a co-administration of polyclonal antibodies against TNFα and IL-1α (T/IL; 100 mg·kg^{-1} i.v., crossed column, $n = 7$) or one polyclonal antibody directed against both cytokines (T+IL; both 30 mg·kg^{-1} i.v., stars or squared column, $n = 7$) at 30 min prior to LPS. Separate group of rats received vehicle (saline) rather than LPS (C; open column, $n = 4$). Data are expressed as mean+s.e.mean of n observations. $P > 0.05$ represents significant difference when compared to LPS-controls at the same time point.

co-administration of two polyclonal antibodies against TNFα and IL-1β (both 30 mg · kg^{-1}, i.v.) or (iv) a polyclonal antibody which neutralises the effects of both TNFα and IL-1β (100 mg · kg^{-1}, i.v.) significantly attenuated the delayed (e.g. after 240 min) fall in MAP (*Fig. 2*). Endotoxaemia for 360 min was also associated with an moderate increase in heart from 402 ± 17 to 455 ± 19 beats min^{-1}, which was not significantly affected by any of the therapeutic interventions used (data not shown).

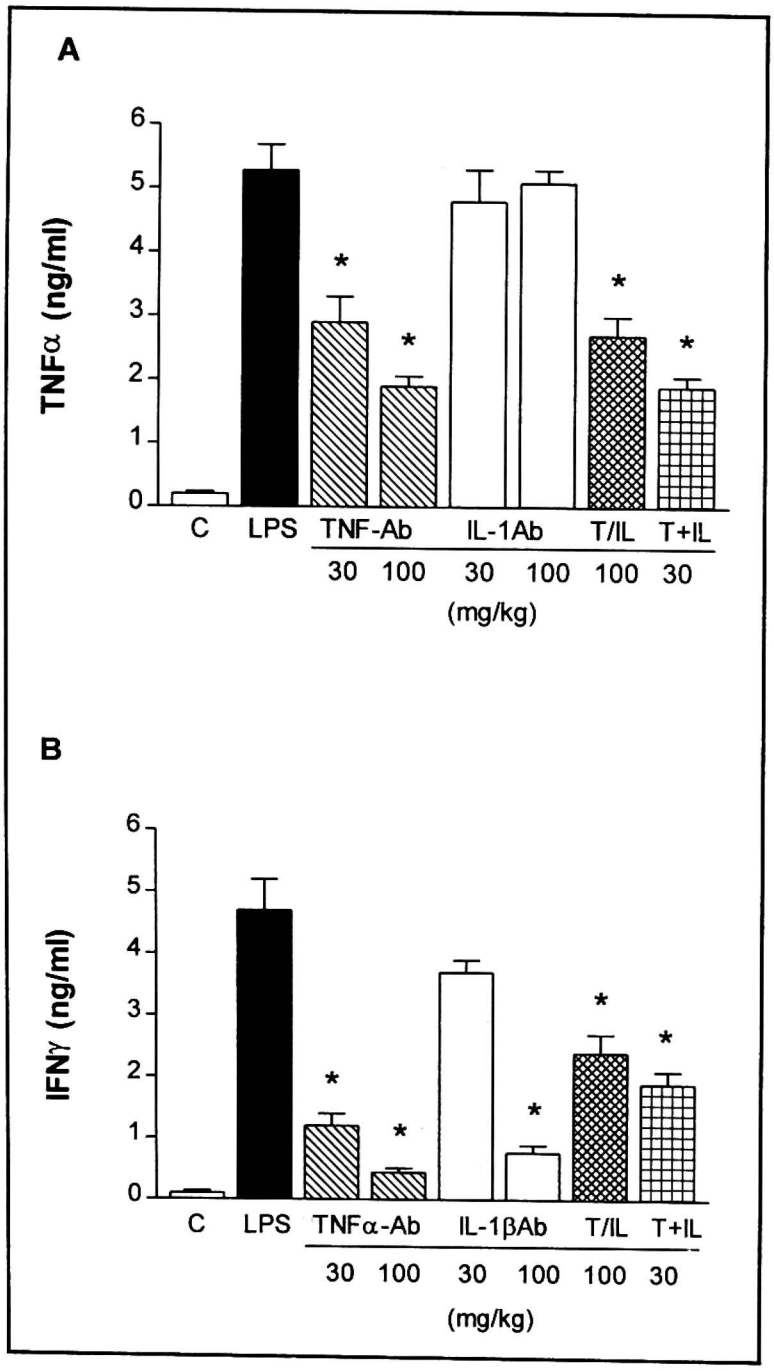

Fig. 3. Effects of (i) a polyclonal antibody against TNFα, (ii) a polyclonal antibody against IL-1, (iii) co-administration of polyclonal antibodies against TNFα and IL-1 and (iv) neutralisation of the effects of both TNFα and IL-1 with one polyclonal antibody directed against both cytokines on the serum concentrations of (A) TNFα at 90 min and (B) IFN at 360 min after LPS (10 mg kg^{-1}, i.v. at time 0; $n = 6$—12 per group). Different groups of LPS-rats received injection of vehicle (saline, 0.6 ml kg^{-1} h^{-1}, solid columns, $n = 12$), a polyclonal antibody against TNFα (TNFα-Ab; 30 or 100 mg kg^{-1} i.v., hatched columns, $n = 6$ per group), a polyclonal antibody against IL-1β (IL-1βAb; 30 or 100 mg kg^{-1} i.v., stippled columns, $n = 6$ per group), co-administration of polyclonal antibodies against TNFα and IL-1β (T/IL; 100 mg kg^{-1} i.v., crossed column, $n = 7$) one polyclonal antibody directed against both cytokines (T+IL; both 30 mg kg^{-1} i.v., squared column, $n = 7$) at 30 min prior to LPS. Separate group of rats received vehicle (saline) rather than LPS (C; open column, $n = 4$). Data are expressed as mean + s.e.mean of n observations. $P > 0.05$ represents significant difference when compared to LPS-controls at the same time point.

Effects of polyclonal antibodies against TNFα and/*or IL-1β on the rises in the serum concentrations of TNFα and IFN caused by endotoxin in the rat*

The maximum increase in the serum concentration of TNFα caused by LPS in the rat (this model) occurs at 90 min after LPS (26), while the maximum

increase in the serum concentration of IFNγ occurs at 300—360 min after injection of LPS (27). Thus, we have investigated the effects of the polyclonal antibodies used in this study (or their combination) on the maximal increase in the serum concentrations of TNFa (at 90 min) and IFNg (at 360 min). Administration of LPS caused a substantial increase in the serum level of TNFα at 90 min ($P>0.05$, $n=12$), which was significantly inhibited by pretreatment of LPS-rats with (i) the polyclonal antibody against TNFα, (ii) co-administration of two polyclonal antibodies against TNFα and IL-1β (both 30 $mg \cdot kg^{-1}$, i.v.) or (iii) a polyclonal antibody which neutralises the effects of both TNFα and IL-1β (100 $mg \cdot kg^{-1}$, i.v.). In contrast, the polyclonal antibody against IL-1β did not affect the rise in the serum levels of TNFα (*Fig. 3a*). Endotoxaemia for 360 min was also associated with a substantial increase in the serum concentrations of IFNγ at 360 min ($P>0.05$, $n=12$), which was significantly attenuated by all of the polyclonal antibodies (or their combination) used (*Fig. 3b*).

Effects of polyclonal antibodies against TNFα and/or IL-1β on the multiple organ dysfunction syndrome caused by endotoxin in the rat

Endotoxaemia for 360 min was associated with a significant increase in the serum levels of the aminotransferases ALT and AST as well as bilirubin (*Fig. 4*). The rise in the serum concentration of AST, ALT and bilirubin was abolished by pretreatment of LPS-rats with (i) the polyclonal antibody against TNFα, (ii) co-administration of two polyclonal antibodies against TNFα and IL-1β (both 30 $mg \cdot kg^{-1}$, i.v.) or (iii) a polyclonal antibody which neutralises the effects of both TNFα and IL-1β (100 $mg \cdot kg^{-1}$, i.v.) (*Fig. 4a, d, e*). In contrast, pre-treatment of LPS-rats with a polyclonal antibody against IL-1β significantly reduced the rise in the serum levels of ALT and AST, but had no effect on the rise in the serum concentration of bilirubin (*Fig. 4c, d, e*). In addition, endotoxaemia for 360 min also caused a significant increase in the serum levels of creatinine and urea, which were not affected by pretreatment of LPS-rats with any of the polyclonal antibodies (or their combination) used (*Fig. 4 a+b*). Pretreatment of LPS-rats with (i) the polyclonal antibody against TNFα, (ii) co-administration of two polyclonal antibodies against TNFα and IL-1β (both 30 $mg \cdot kg^{-1}$, i.v.) or (iii) a polyclonal antibody which neutralises the effects of both TNFα and IL-1β (100 $mg \cdot kg^{-1}$, i.v.), however, attenuated the rise in the serum levels of lipase caused by 360 min of endotoxaemia. In contrast, the polyclonal antibody against IL-1β did not affect the rise in the serum levels of lipase caused by endotoxin (*Fig. 4f*).

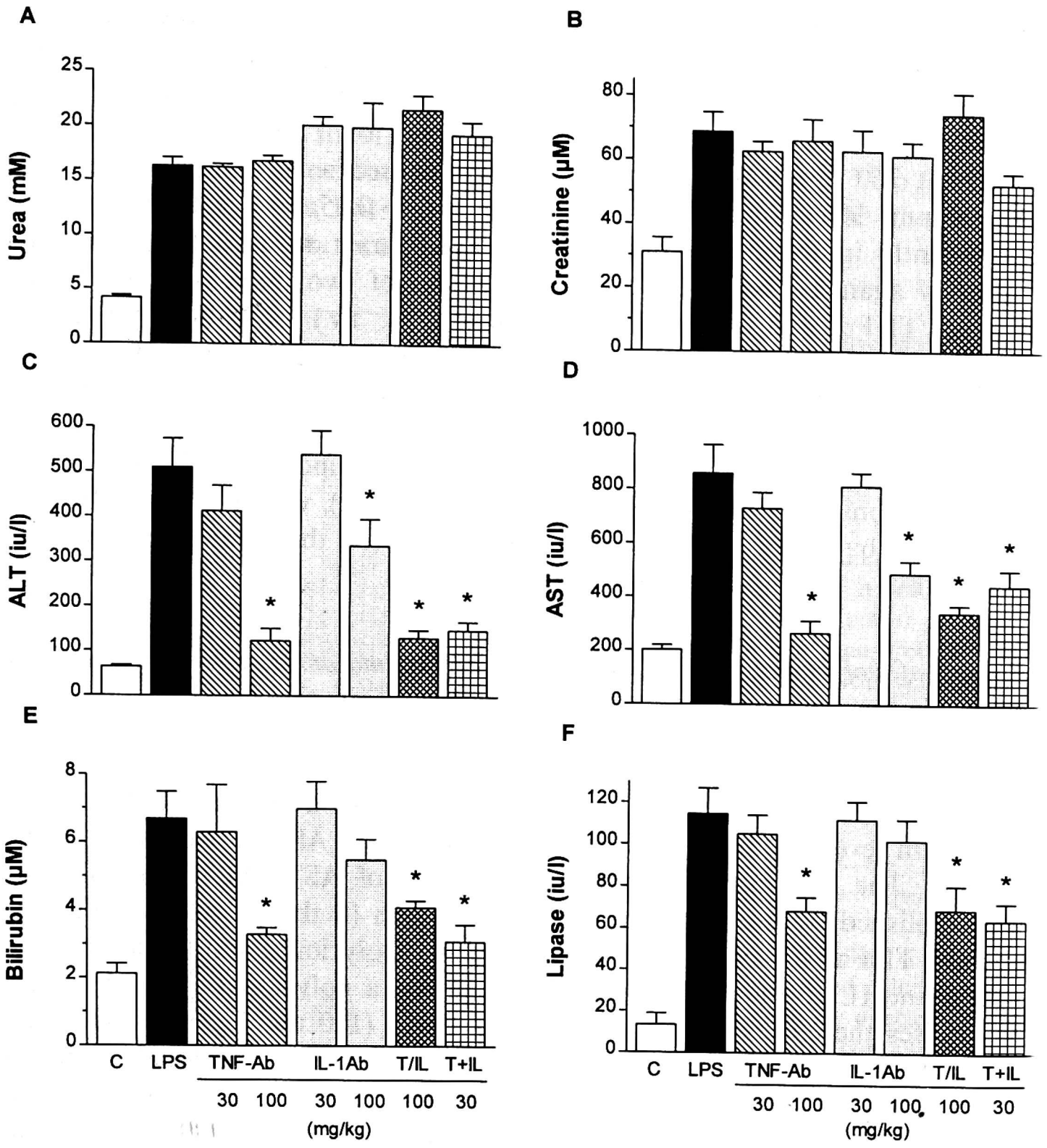

Fig. 4. Effects of (i) a polyclonal antibody against TNFα, (ii) a polyclonal antibody against IL-1, (iii) co-administration of polyclonal antibodies against TNFα and IL-1 and (iv) neutralization of the effects of both TNFα and IL-1 with one polyclonal antibody directed against both cytokines on the LPS-induced increases in the serum concentrations of urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and lipase. Biochemical markers of organ dysfunction were measured in serum obtained from rats treated with vehicle rather than LPS or rats treated with *E. coli* LPS (10 mg·kg^{-1}, i.v.) for 6 h. Different groups of LPS-rats received injection of vehicle (saline, 0.6 ml·kg^{-1} h^{-1}, solid columns, $n = 12$), a polyclonal antibody against TNFα (TNFα-Ab; 30 or 100 mg·kg^{-1} i.v., hatched columns, $n = 6$ per group), a polyclonal antibody against IL-1β (IL-1βAb; 30 or 100 mg·kg^{-1} i.v., stippled columns, $n = 6$ per group), co-administration of polyclonal antibodies against TNFα and IL-1β (T/IL; 100 mg·kg^{-1} i.v., crossed column, $n = 7$) one polyclonal antibody directed against both cytokines (T+IL; both 30 mg·kg^{-1} i.v., squared column, $n = 7$) at 30 min prior to LPS. Separate group of rats received vehicle (saline) rather than LPS (C; open column, $n = 4$). Data are expressed as mean+s.e.mean of n observations. $P > 0.05$ represents significant difference when compared to LPS-controls at the same time point.

Effects of polyclonal antibodies *against TNFα and/or IL-1β on the increase in iNOS activity in lung and liver of rats with endotoxic shock*

Endotoxaemia for 360 min was associated with a substantial increase in iNOS activity in lung and liver homogenates ($P > 0.05$, *Fig. 5*). The activity of iNOS was significantly reduced in homogenates of lung and liver obtained from LPS-rats treated with significantly inhibited by pretreatment of LPS-rats with (i) the polyclonal antibody against TNFα, (ii) the polyclonal antibody against IL-1β, (iii) co-administration of two polyclonal antibodies against TNFα and IL-1β or (iv) a polyclonal antibody which neutralises the effects of both TNFα and IL-1β (*Fig. 5*).

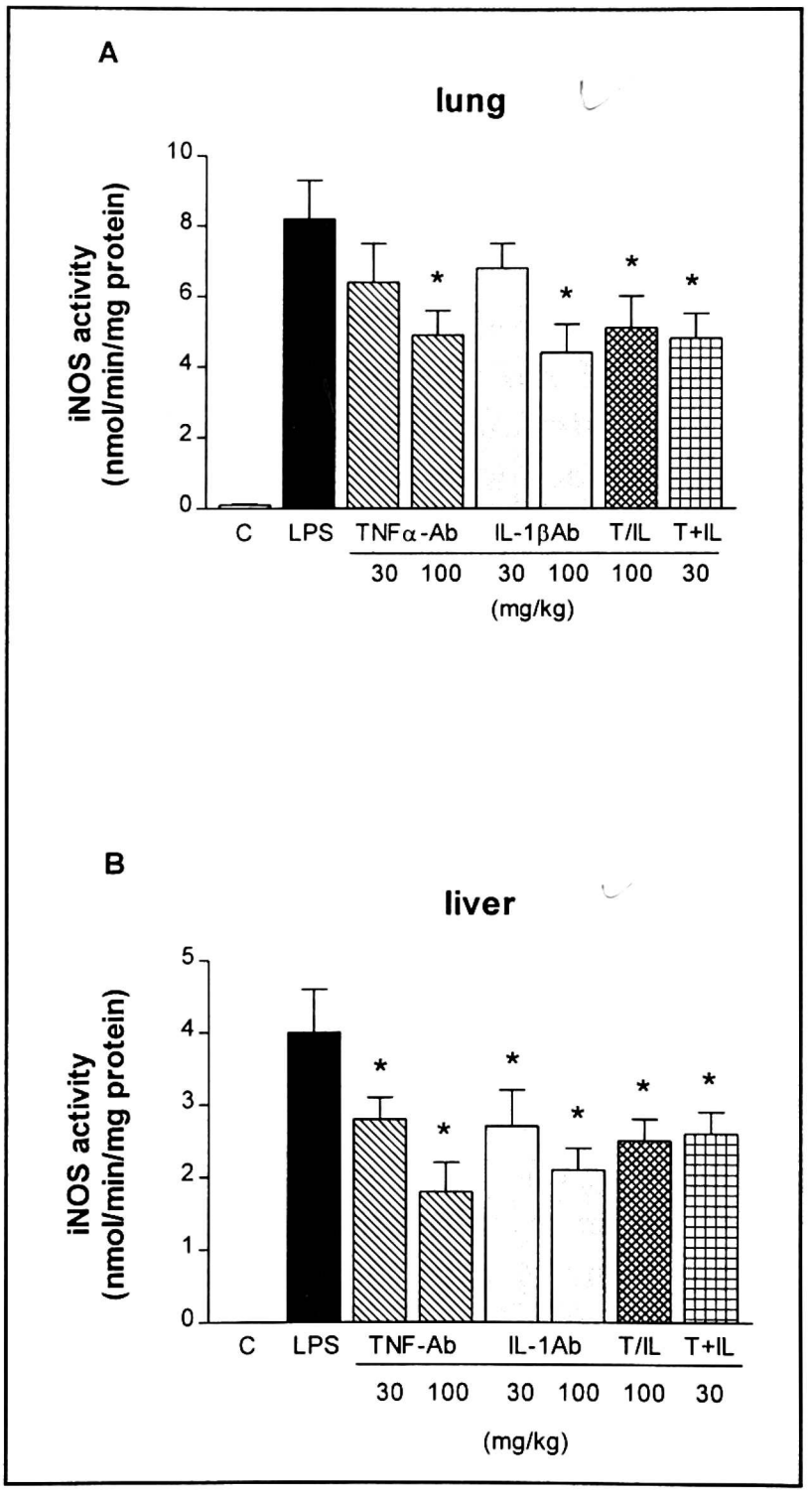

Fig. 5. Pre-treatment of rats with (i) a polyclonal antibody against TNFα, (ii) a polyclonal antibody against IL-1, (iii) two separate, polyclonal antibodies against TNFα and IL-1 and (iv) one polyclonal antibody directed against both cytokines attenuates the induction of a calcium-independent iNOS activity in (A) lung and (B) liver homogenates. Calcium-independent iNOS activity was measured in lung and liver homogenates obtained from rats infused with vehicle rather than LPS (control, C, open columns, $n = 4$) or rats treated with *E. coli* LPS (10 mg·kg^{-1}, i.v., $n = 12$). Different groups of LPS-rats received injection of vehicle (saline, 0.6 ml kg^{-1} h^{-1}, solid columns, $n = 12$), a polyclonal antibody against TNFα (TNFα-Ab; 30 or 100 mg·kg^{-1} i.v., hatched columns, $n = 6$ per group), a polyclonal antibody against IL-1β (IL-1βAb; 30 or 100 mg·kg^{-1} i.v., stippled columns, $n = 6$ per group), co-administration of polyclonal antibodies against TNFα and IL-1β (T/IL; 100 mg·kg^{-1} i.v., crossed column, $n = 7$) one polyclonal antibody directed against both cytokines (T+IL; both 30 mg·kg^{-1} i.v., squared column, $n = 7$) at 30 min prior to LPS. Data are expressed as mean+s.e.mean of n observations. *$P > 0.05$ represents significant difference when compared to LPS-controls at the same time point.

DISCUSSION

The circulatory failure caused by endotoxin in the rat is due to an enhanced formation of NO (28) due to an (early) activation of eNOS and a delayed induction of iNOS in the vasculature (29—31). Interestingly, both the circulatory failure as well as the induction of iNOS are attenuated by pre-treatment of rats with either a polyclonal antibody against TNFα (32) or the endogenous IL-1 receptor antagonist (33). Thus, an enhanced formation of endogenous TNFα and IL-1 contribute to the induction of iNOS and the circulatory failure caused by endotoxin in the rat. Here we confirm that polyclonal antibodies against either TNFα or IL-1 attenuate the circulatory failure as well as the induction of iNOS caused by endotoxin in the rat. The progression of shock to multiple organ failure (or MODS) is associated with an increase in the mortality so that with the number of organs failing (from 1—4) mortality progressively increases from 30% (in the absence of MODS) to 100% (22, 34). In the rat model of endotoxic shock used here, six hours of endotoxaemia resulted in a substantial increase in the plasma levels of bilirubin, ALT and AST indicating the development of acute liver injury/dysfunction. Pre-treatment of rats with either the polyclonal antibody against TNFα or the polyclonal antibody against IL-1 attenuated the acute liver injury/dysfunction caused by endotoxin. Endotoxaemia also resulted in an increase in the serum activity of lipase, an indicator of pancreatic injury, which was also attenuated by pre-treatment of rats with either the polyclonal antibody against TNFα or the polyclonal antibody against IL-1. However neither of these antibodies effected the rise in urea or creatinine caused by endotoxin. These findings strongly suggest that an enhanced formation of TNFα and IL-1 contributes to the circulatory failure, the liver injury/dysfunction (8, 9, this study), the pancreatic injury, but not the renal dysfunction caused by endotoxin in the rat.

In the rat, endotoxin causes an overproduction of NO due to induction of iNOS (29—31) which contributes to the circulatory failure (28—31) the liver injury/dysfunction as well as the pancreatic injury caused by endotoxin (35, 36). We demonstrate here that pre-treatment of rat aortic smooth muscle cells (prior to the activation with either TNFα or IL-1) or of LPS-rats with either a polyclonal antibody against TNFα or a polyclonal antibody against IL-1 significantly attenuates the increase in iNOS activity (measured as the formation of nitrite/nitrate) in the cell supernatant. Similarly pretreatment of rats with these antibodies prevented the rise in the serum levels of nitrite/nitrate caused by endotoxin in the rat. Thus, we propose that a reduction in the expression of iNOS protein and activity contributes to the reduction by these antibodies of the circulatory failure and the organ dysfunction and injury caused by LPS in the rat. This conclusion

is also supported by the finding that neither of the polyclonal antibodies used in this study — like several chemically distinct inhibitors of iNOS activity (35, 36) or other agents which prevent the expression of iNOS proteins such as dexamethasone or calpain inhibitor I (37) — attenuates the injury/dysfunction of liver and pancreas (but not the renal dysfunction) caused by endotoxin in the rat.

There is now a substantial amount of evidence that interventions aimed at preventing the effects of either TNFα or IL-1 reduce the circulatory failure, the organ dysfunction and/or injury as well as the mortality caused by endotoxin or bacteria in animals. In contrast, clinical trials aimed at demonstrating a reduction in 28-day mortality with such interventions have so far not met with the expected success. For instance, there is no convincing evidence that interventions aimed at reducing the effects of TNFα (e.g. antibodies against TNFα, soluble TNFα receptors etc) cause a significant reduction in 28-day mortality in patients with septic shock (38—41). Most notably, there is one recent report documenting that the treatment of septic patients with the TNF receptor:Fc fusion protein causes a dose-related increase in mortality (42). Similarly, clinical trials evaluating the effects of the IL-1 receptor antagonists have not resulted in a significant reduction in 28-day mortality (43—46). Although the above trials failed to provide evidence that any of the anti-cytokine interventions used caused a significant reduction in 28-mortality, these studies nevertheless support the view that both TNFα as well as IL-1 play a role in the pathophysiology of septic shock and indicate that anticytokine therapy may well be of benefit for certain groups of patients. The IL-1ra Phase III Sepsis Syndrome Group has recently reported that (i) there is a direct relationship between a patient's Predicted Risk of Mortality at study at entry and the efficacy of the IL-1 receptor antagonist (Il-1ra) in that (ii) patients with a Predicted Risk of Mortality of $>24\%$ derived little benefit, while (iii) IL-1ra reduced the risk of death in the first 2 days for patients with a Predicted Risk of Mortality of $< 24\%$ (46).

The reasons for the discrepancy in outcome between animal experiments and clinical trials are not entirely clear, but may include (i) relatively late intervention in clinical trials (vs. pre-treatment in animal studies), (ii) inhomogeneity of patients (e.g. differences in age, gender, causes of shock, severity of disease) or (iii) the pharmacology (dose regimen, time of intervention, length of treatment) of the intervention chosen. One could also argue that the pathophysiology leading to the circulatory failure, organ dysfunction and ultimately death in patients with septic shock is multifactorial and, hence, that interventions aimed at eliminating the detrimental effects of a single mediator („single-bullet approach to the therapy of shock") — although useful in some acute animal models — are less likely (if not unlikely) to cause a significant reduction in 28-day mortality. Indeed, there is

some evidence that the prevention of the formation of both TNFα and IL-1β (e.g. with interferon- or IL-10) is superior to prevention of the formation of either one of these cytokines in reducing mortality in rodent models of endotoxaemia (47). Moreover, the reduction in survival afforded by a combination immunotherapy (antibody against TNFα, J5 antiserum against endotoxin and a *Pseudomonas* O-serotype-specific opsonophagocytic monoclonal antibody) was greater than the one afforded by any combination of two antibodies or single antibody therapy (48). We demonstrate here that (i) co-administration of two polyclonal antibodies directed against either TNFα or IL-1 or (ii) neutralisation of the effects of either TNFα or IL-1 with one polyclonal antibody directed against both cytokines is superior in reducing the circulatory failure and MODS caused by endotoxin in the rat than a therapy with a single antibody directed against either cytokine. Thus, (i) TNFα and IL-1 contribute to the circulatory failure and MODS caused by endotoxin in either an additive or synergistic manner, and (ii) a combination immunotherapy which neutralises the effects of both TNFα and IL-1β may be superior in the treatment of circulatory failure and MODS caused by endotoxin than a single antibody therapy. Having stated above that some anticytokine therapies have caused an increase in mortality in patients with septic shock (46), it should also be noted that there may be potential hazards of combination immunotherapy. For instance, co-administration of IL-1ra and TNF-binding protein caused an increase in mortality in neutropenic rats with sepsis caused by Pseudomonas aeruginosa (49). Thus, further studies are warranted to gain a better understanding of the beneficial and adverse effects of combination immunotherapy in experimental endotoxaemia and sepsis.

In conclusion, this study demonstrates that (i) a polyclonal antibody against TNFα, (ii) a polyclonal antibody against IL-1, (iii) co-administration of polyclonal antibodies against TNFα and IL-1 and (iv) neutralisation of the effects of both TNFα and IL-1 with one polyclonal antibody directed against both cytokines reduces the circulatory failure, the liver injury/dysfunction, the pancreatic injury (but not the renal dysfunction caused by endotoxin in the rat). The beneficial effects of these interventions on haemodynamics and organ injury/dysfunction are most likely due to prevention of the induction of iNOS. We also report that two different interventions which neutralise the effects of both TNFα and IL-1 were superior in reducing the circulatory failure and the organ injury caused by endotoxin in the rat, than single interventions aimed at neutralising the effects of either cytokine. Thus, we propose that interventions which are able to neutralise the effects of both TNFα and IL-1 (combination immunotherapy) may be of benefit in the treatment of patients with septic shock.

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