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INVOLVEMENT OF THE LIVER, BUT NOT OF IL-6, IN IL-1-INDUCED DESENSITIZATION TO THE LETHAL EFFECTS OF TUMOR NECROSIS FACTOR[1]

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C57BL/cnb mice were found to be protected against a lethal combination of recombinant murine (m) TNF and GalN by pretreatment with several cytokines. At certain doses, rmTNF and human (h) TNF protected completely. The clearest protection was induced by rIL-1: all four rIL-1 species (both m and h, as well as α and β) protected when given 12 h before the challenge. LPS and rmIFN-γ protected weakly, whereas rmIL-6 and rhIL-6 did not protect at all. Also adrenocorticotropic hormone, dexamethasone, or dexamethasone in combination with rhIL-6 could not protect. A single IL-1 injection also completely protected mice against a lethal dose of mTNF in the absence of GalN sensitization. The desensitization by IL-1 cannot be explained by a faster clearance of the challenge TNF. In addition, we demonstrate that the IL-1-induced desensitization was only observed when a functioning liver was present, that IL-1-pretreated animals did not show decreased numbers of hepatocyte TNF receptors, and that the amount of TNF-induced IL-6 was not reduced.

During the last decade, it has become clear that endotoxic shock induced by bacteria or their cell wall components is mediated by monokines (1). In this respect, TNF has proven to be the major responsible mediator. The most important evidence has come from Beutler et al. (2), who were able to reduce lethality of LPS in mice and baboons by pretreatment with a non-LPS-reactive anti-TNF antibody. However, two other monokines, namely, IL-1 and IL-6, have also been found in the serum of septic shock patients (3); it is not known yet to what extent these two monokines play a role in the pathology.

In order to apply the antitumor properties of TNF for therapy, a reduction of the harmful effects of TNF is required, without affecting its antitumor activity. Several drugs have been reported to protect against the lethal effects of TNF (4). Protection, without loss of antitumor activity, was seen with the cyclooxygenase inhibitor indomethacin (5). Furthermore, several lines of evidence indicate that rescue and feedback regulatory systems are activated in vivo. IL-1 and IL-6, both induced by LPS and TNF, can in turn induce the release of ACTH[3] (6), leading to elevated levels of glucocorticoids. DEX, a glucocorticoid analogue, has proven to be effective in counteracting the toxic effects of both LPS and TNF[4] (7), at least when given shortly before. Wallach et al. (8) have shown that animals pretreated with hTNF or hIL-1α survived a lethal challenge of hTNF or hIL-1α in combination with ActD or GalN. It was suggested that TNF and IL-1 induce (a) protective system(s), an activity which is suppressed by GalN and/or ActD. GalN is a specific hepatotoxic agent that exerts its activity by impairing the macromolecular synthesis in hepatocytes through a depletion of UTP.

In this study, we repeated these desensitization protocols and extended them to a challenge with rmTNF alone (without GalN) and to pretreatment with other cytokines, ACTH, and glucocorticoids. In addition, we studied some possible mechanisms of this desensitization, such as the clearance of challenging TNF, the effects on TNFR, and the involvement of the liver. Finally, we investigated a possible role for IL-6, a cytokine induced by both TNF and IL-1, of which the main target organ is the liver.

MATERIALS AND METHODS

Laboratory animals. Female C57BL/cnb mice (SCK, Mol, Belgium) were used at the age of 7–9 wk (18–20 g). The animals were kept in 12-h light/dark cycles in a temperature-controlled, air-conditioned room, and received food and water ad libitum.

Reagents and injections. GalN, Urd, and ACTH were obtained from Sigma Chemical Co., St. Louis, MO. *Escherichia coli* 0111:B4 LPS was from Difco Laboratories, Detroit, MI. DEX (Decadron) was purchased from Merck Sharpe & Dohme, St. Louis, MO. RU486 was a generous gift from Dr. D. Martini, Roussel UCLAF, Romainville, France. Purified rhTNF and rmTNF were obtained from Dr. J. Tavernier (formerly of Biogent, Gent, Belgium). rhTNF had a sp. act. of 2.5×10^7 U/mg and contained less than 0.11 ng of endotoxin/mg of protein; rmTNF had a sp. act. of 7.5×10^7 U/mg and contained less than 0.96 ng of endotoxin/mg of protein. The four rIL-1 preparations (rmIL-1α, rmIL-1β, rhIL-1α, and rhIL-1β) were prepared in the Glaxo Institute for Molecular Biology, Geneva, Switzerland. Their endotoxin contamination did not exceed 3.85, 0.35, 1.40, and 0.30 ng/mg protein, and their sp. act. (as tested in a lymphocyte-activating factor assay) amounted to 2.8×10^7, 2.0×10^7, 2.4×10^7, and $1.1 \times$

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[3] Abbreviations used in this paper: ACTH, adrenocorticotropic hormone; ActD, actinomycin D; DEX, dexamethasone; GalN, galactosamine; h, human; m, murine; Urd, uridine; UTP, uridine triphosphate; PBSA, Ca^{2+}- and Mg^{2+}-free PBS.

[4] Libert, C., P. Brouckaert, and W. Fiers. 1991. The influence of modulating substances on tumor necrosis factor and interleukin 6 levels after injection of murine tumor necrosis factor or lipopolysaccharide in mice. *Submitted for publication.*

10^7 U/mg, respectively. rhIL-6 was cloned in and purified from yeast in our laboratory to a sp. act. of 2×10^8 U/mg (Y. Guisez, J. Demolder, and R. Contreras, unpublished data); rhIL-6 contained less than 0.7 ng of endotoxin/mg of protein. rmIL-6 (sp. act. 8×10^8 U/mg) was a kind gift from Dr. J. Van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium). rmIFN-γ was cloned in and purified from *E. coli* in our laboratory; the purified material had a sp. act. of 1.7×10^6 U/mg and contained less than 10.1 ng of endotoxin/mg of protein. The reagents were diluted in endotoxin-free PBS before injection. The injections were given i.v. (lateral tail vein) or i.p., as indicated. All challenging injections were given between 9 and 10 a.m.

TNF and IL-6 determinations in serum. TNF was assayed by using the sensitive cell line WEHI 164 cl 13 (9). Briefly, WEHI cells (50,000 cells/culture well) were cultured with ActD (1 μg/ml) in the presence of diluted serum samples in a flat-bottomed 96-well microtiter plate. After 18 h of incubation, the numbers of surviving cells were determined using the MTT colorimetric method. Several rTNF preparations were included as standards. The detection limit of the assay varied between 0.2 and 0.5 pg/ml.

IL-6 was determined by means of IL-6-dependent 7TD1 hybridoma cells (10). Seven thousand cells/well were cultured in 96-well plates in the presence of serial dilutions of serum, or rmIL-6 as a standard. After 3 days, the number of cells was determined by the hexosaminidase colorimetric method. The detection limit of the assay was about 1 pg/ml. In previous experiments we demonstrated the specificity of the assay by using a mAb against rmIL-6 (11).

Isolation of hepatocytes and preparation of membranes. Hepatocytes were obtained according to the method of Soley and Hollenberg (12). Briefly, after anesthesia, the upper vena cava posterior was cannulated and retrograde perfusion (10 ml/min at 37°C) was started with Ca^{2+}/Mg^{2+}-free HBSS containing 0.5 mM EGTA, pH 7.3. The lower vena cava posterior was tied off and the perfusion fluid was allowed to escape via the portal vein. After 5 min, the perfusion fluid was changed to a collagenase (0.5 mg/ml; Cooper Biomedical, Malvern, PA) digestion buffer containing NaCl (136.8 mM), Na_2HPO_4 (1.33 mM), KCl (5.4 mM), KH_2PO_4 (0.44 mM), $MgCl_2$ (0.98 mM), $MgSO_4$ (0.8 mM), $CaCl_2$ (5 mM), glucose (5.5 mM), and HEPES (50 mM). The perfusion (20 ml/min at 37°C) was continued for 5 min. The liver was then excised and transferred to a petri dish containing 15 ml of the same buffer, but without collagenase. The liver was teased apart gently, after which the released cells were collected and cleaned by three cycles of centrifugation ($50 \times g$ for 3 min at 4°C) and resuspension. For the preparation of membranes, 5×10^7 cells were resuspended in 2 ml of an ice-cold hypotonic buffer: 10 mM Tris, 1 mM PMSF (Sigma), and 100 KIU/ml aprotinin (Bayer, Werk Elberfeld, FRG), pH 7.4. The cells were broken by using a Dounce homogenizer and nuclei were removed by two centrifugations at $2,000 \times g$ (4°C, 15 min). The supernatant was then centrifuged for 15 min at $20,000 \times g$ (4°C). The pelleted membranes were carefully washed with 200 μl of PBSA containing 1 mM PMSF and 100 KIU/ml aprotinin. Protein concentrations were determined by the method of Bradford (13).

Iodination of mTNF and binding studies. mTNF was radioiodinated by means of the Iodogen method (Pierce Chemical Co., Rockford, IL) to a sp. act. of 7×10^7 cpm/μg. After iodination, TNF had retained 50% of its specific biologic activity and after phenol extraction was shown to contain less than 7% free iodine.

The binding experiments were performed as follows. Triplicates of 20 μg of the membrane suspensions were incubated with 4 ng of iodinated TNF ([^{125}I]TNF) in 160 μl of PBSA, containing 0.1% BSA. Nonspecific binding was determined, also in triplicate, in the presence of a 500-fold excess of unlabeled mTNF. The TNF was allowed to bind for 4 h at 4°C, after which the membranes were centrifuged for 15 min at $16,000 \times g$ to separate bound TNF from unbound TNF. The pellet was washed twice with PBSA containing 0.1% BSA, resuspended in 200 μl of the same buffer, and counted in a gamma counter (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Tissue distribution of TNF. For each animal, 0.5×10^6 cpm (approximately 7 ng) of [^{125}I]TNF was added to 10 μg of rmTNF and injected i.v. Thirty minutes or 6 h after the injection, the animals were killed by cervical dislocation. The different organs were isolated and their net weights, as well as the incorporated radioactivity, were determined. For each particular organ, the radioactivity was recalculated to that of a standard-sized organ by dividing the radioactivity by the net organ weight and multiplying by the mean net weight of the organ of six mice at this time point. The mean weights (in mg) at 30 min were 117 ± 43 (skin), 771 ± 73 (liver), 127 ± 8 (kidney), 840 ± 235 (intestine), 138 ± 61 (spleen), 425 ± 113 (stomach), 167 ± 71 (heart), and 148 ± 51 (lung), and those at 6 h were 86 ± 20 (skin), 707 ± 58 (liver), 139 ± 13 (kidney), 809 ± 229 (intestine), 129 ± 27 (spleen), 454 ± 139 (stomach), 126 ± 28 (heart), and 179 ± 14 (lung). As measured by Student's *t* test, no significant changes were found for the weights at 30 min vs 6 h.

Determination of body temperature. The rectal temperature was measured with an electronic thermometer (Comark Electronics, Littlehampton, UK; model 2001).

RESULTS

Protection against mTNF/GalN lethality by several cytokines. All control animals (whether untreated or PBS-pretreated) that received 0.5 μg of mTNF in combination with 20 mg of GalN died about 12 h after the injection. We tested the protective properties of several cytokines, when given 12 h before this challenge (Table I). mIL-1α-pretreated animals all survived; mTNF as well as hTNF at certain doses (1 μg and 5 μg, respectively) showed clear protection (100% and 90%, respectively); both LPS and mIFN-γ largely prevented rapid killing, but eventually they could not fully protect at the doses tested. We previously reported that mTNF is considerably more toxic (about 50-fold) to mice than hTNF (14); this species specificity, however, largely disappears in the sensitization system involving GalN. For rmTNF, the LD_{50} is about 10 μg per animal, whereas for rhTNF, several hundred micrograms are required for induction of lethality. This is the reason why higher (sublethal) doses of rmTNF no longer protected, whereas in the case of rhTNF, a straight dose response was observed.

Data concerning pretreatment with IL-6, both murine and human, are shown in Table II. IL-6, given as a single injection, was not able to induce protection against TNF/GalN lethality at the doses tested. Also, continuous exposure to IL-6 by means of a 4-h i.v. infusion, which mimicks IL-1-induced IL-6 better than a bolus injection, could not desensitize. To detect a possible involvement of glucocorticoids, we treated the mice with ACTH, either at 0.1 μg/g or 1 μg/g (15), or with DEX at 750 μg (7) per animal. None of these agents could, however, protect (Table II). When DEX was given in combination with rhIL-6, a mixture reported to be most effective in inducing acute phase protein genes (16), no protection was observed. Also, when RU486, a synthetic glucocorticoid

TABLE I

Effect of pretreatment with different cytokines on the lethality induced by rmTNF + GalN

Pretreatment[a]	Dose	Lethality[b]	
		12 h	72 h
		10/10	10/10
PBS		10/10	10/10
rmIL-1α	1 μg	0/5	0/5
	0.5 μg	0/5	0/5
rmTNF	5 μg	5/10	8/10
	1 μg	0/10	0/10
	0.5 μg	1/5	3/5
	0.1 μg	4/5	5/5
rhTNF	5 μg	0/10	1/10
	1 μg	2/10	5/10
	0.5 μg	3/5	4/5
	0.1 μg	5/5	5/5
LPS	10 μg	1/5	3/5
	1 μg	0/5	4/5
	100 ng	0/5	2/5
	10 ng	0/5	5/5
	1 ng	4/5	5/5
	0.1 ng	3/5	5/5
rmIFN-γ	1×10^5 U	1/5	5/5
	2.5×10^3 U	2/5	5/5
	5×10^3 U	1/5	5/5

[a] Pretreatment was given i.v. (0.25 ml) 12 h before the challenge with 0.5 μg of rmRNF + 20 mg of GalN given i.p. (total volume: 0.5 ml).

[b] Number of dead mice vs total number of mice recorded 12 h and 72 h after the challenge injection (no further deaths occurred).

TABLE II

Effect of different doses of IL-6, ACTH, and corticoids on rmTNF + GalN-induced lethality

Pretreatment	Dose	Lethality[a] 12 h	Lethality[a] 72 h
Single injection			
PBS[a]		5/5	5/5
rmIL-6[a]	5 μg	5/5	5/5
	1 μg	5/5	5/5
	0.2 μg	5/5	5/5
rhIL-6[a]	5 μg	10/10	10/10
	1 μg	10/10	10/10
	0.2 μg	5/5	5/5
rhIL-6[b]	5 μg	4/5	5/5
	1 μg	4/5	5/5
ACTH[a]	2 μg	5/5	5/5
	20 μg	4/5	5/5
DEX[a]	750 μg	5/5	5/5
DEX	750 μg + rhIL-6 1 μg[a]	4/5	5/5
DEX	750 μg + rhIL-6 5 μg[a]	5/5	5/5
Infusion[c]			
PBS	80 μl/h	5/5	5/5
rhIL-6	0.25 μg/h	5/5	5/5
	1.25 μg/h	5/5	5/5
rhIL-1	0.25 μg/h	0/5	0/5

[a] Pretreatment was given i.v. (0.25 ml) 12 h before the challenge (0.5 ml i.p. in all experiments) with 0.5 μg of rmTNF + 20 mg of GalN.
[b] Pretreatment was given i.v. (0.25 ml) 8 h before the challenge.
[c] Pretreatment was given i.v. (0.32 ml) over 4 h, between 12 and 8 h before the challenge.

TABLE III

Protection by rmTNF or rmIL-1α pretreatment against lethality induced by rmTNF, rhTNF, or LPS in combination with GalN

Challenge	Pretreatment[a]/Lethality[b] –	PBS	1 μg rmTNF	1 μg rmIL-1α
0.5 μg rmTNF + 20 mg GalN	5/5	5/5	0/5	0/5
0.5 μg rhTNF + 20 mg GalN	5/5	5/5	0/5	0/5
50 ng LPS + 20 mg GalN	5/5	5/5	0/5	0/5

[a] Pretreatment was given i.v. (0.25 ml) 12 h before the challenge (total volume: 0.5 ml).
[b] Number of dead mice vs total number of mice recorded after 24 h (no further deaths occurred).

receptor blocker, was added, no inhibition of the IL-1-induced protection was observed (data not shown). Finally, we administered rhIL-6 at the same time with varying doses of rhIL-1β, and studied the effects on protection: IL-6 did not affect the IL-1-induced protection negatively or positively (results not shown).

In Table III, we demonstrate that mTNF and mIL-1α both protected against mTNF/GalN, hTNF/GalN, and LPS/GalN lethality. In other words, for TNF, cross-protection between hTNF and mTNF was observed.

Protection is induced by four different IL-1 species. We observed that four IL-1 species, namely, mIL-1α, mIL-1β, hIL-1α, and hIL-1β, equally protected against a lethal challenge with TNF/GalN (data not shown). The protective effect of IL-1 was 100% for 20, 10, 2, and 1 μg, but started to become less effective below 0.5 μg per mouse. Optimal protection was achieved when IL-1 was given 4 to 12 h before the challenge (Table IV). At time intervals of 16 and 20 h, only the higher IL-1 concentrations could desensitize, whereas 2 μg of IL-1, given 24 h before the challenge, no longer protected.

A single injection of IL-1 protects against lethality induced by a single injection of mTNF in the absence of GalN. As shown in Table V, a single i.v. injection of 1 μg of mIL-1α completely prevented lethality induced by 12.5 μg of mTNF. The IL-1 was given 12 h before the

TABLE IV

Time dependency of IL-1-induced protection against TNF + GalN lethality

Time Interval (h) Between Pretreatment (rmIL-1β) and Challenge (mTNF + GalN)	Lethality[a] Control	μg rmIL-1β 0.5	1	2
−2	5/5	ND	5/5	ND
0	5/5	ND	5/5	ND
2	5/5	ND	5/5	ND
4	ND	ND	0/5	ND
8	5/5	0/5	2/5	0/5
12	5/5	0/5	0/5	0/5
16	ND	2/5	1/5	1/5
20	ND	5/5	4/5	1/5
24	5/5	ND	ND	5/5

[a] Number of dead mice vs total number of mice recorded after 24 h (no further deaths occurred). rmIL-1β at the indicated dose was given i.v. in a volume of 0.25 ml. The challenge, rmTNF (0.5 μg) + GalN (20 mg), was given at $t = 0$ h i.p. in 0.5 ml of PBS.

TABLE V

Protective effect of rmIL-1α on lethality induced by rmTNF

Pretreatment[a]	Dose	Lethality[b]
		5/5
PBS		10/10
rmIL-1α	1 μg	0/10
	2 μg	1/5
	5 μg	1/5
rmTNF	1 μg	3/5

[a] Pretreatment was given i.v. (0.25 ml) 12 h before the challenge with 12.5 μg of rmTNF given i.v. (total volume: 0.25 ml of PBS).
[b] Number of dead mice vs total number of mice recorded 72 h after the challenge injection (no further deaths occurred).

challenge. IL-1 (2 μg and 5 μg) also had a clear effect, whereas mTNF was not very effective.

The animals that survived the lethal challenge nevertheless went through a period of severe illness. This is illustrated in Figure 1, which shows a similar drop in body temperature in the two groups of animals. But whereas the majority of the PBS-pretreated animals were dead 24 h after the TNF injection, the IL-1-treated mice survived and their body temperature returned to normal values. In the same animals (Fig. 1), we determined the levels and pharmacokinetics of TNF and IL-6 (Fig. 2). We observed that the TNF levels in the IL-1-pretreated animals were significantly higher than in the control (PBS-treated) animals (Fig. 2*A*). A similar result was found when [^{125}I]TNF was added to the challenge and radioactivity was measured in the serum (Fig. 2*A*, *inset*). This radioactivity was most probably due to iodinated TNF in mice, because after phenol extraction on selected samples, the activity was shown to be almost entirely (94%) protein-bound. Also, the induced IL-6 levels were higher in IL-1-treated animals as compared with control animals (Fig. 2*B*).

Involvement of the liver in IL-1-induced protection. In Table VI we demonstrate that the protection conferred by IL-1 was markedly reduced by co-administration of GalN, 12 h before the challenge with mTNF/GalN. In these experiments, GalN in combination with up to 2 μg of IL-1 (in the absence of a challenge) never caused sickness or lethality. GalN alone protected very weakly, but this might be an effect of contaminating LPS. It may also be noted that inhibition of the effect of IL-1 by GalN was completely reversed by Urd.

Binding of labeled mTNF to membranes of hepatocytes. Mice were pretreated with either 2 μg of IL-1 or PBS. Twelve hours later, hepatocytes were isolated and

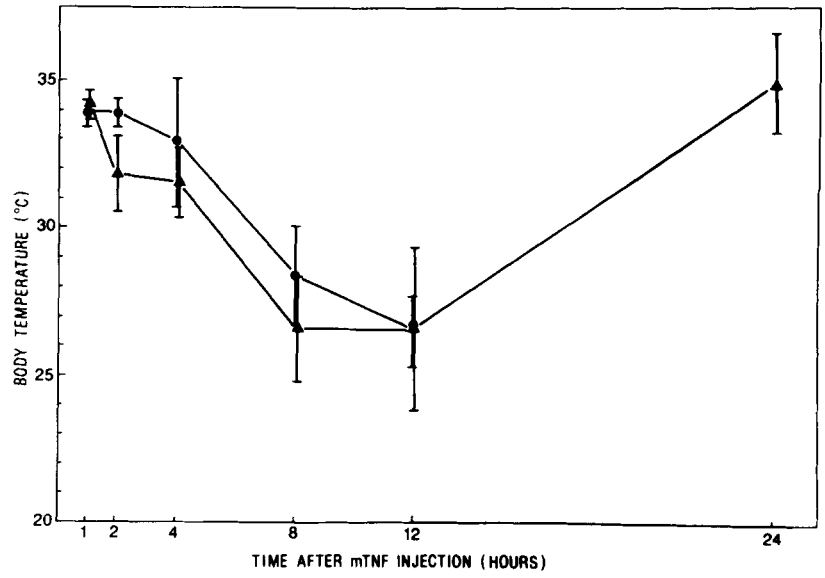

Figure 1. Effect of IL-1 pretreatment on the change in body temperature induced by mTNF. PBS (●) or 1 μg of mIL-1α (▲) was given i.v. 12 h before an i.v. challenge with 12.5 μg of mTNF ($n = 5$).

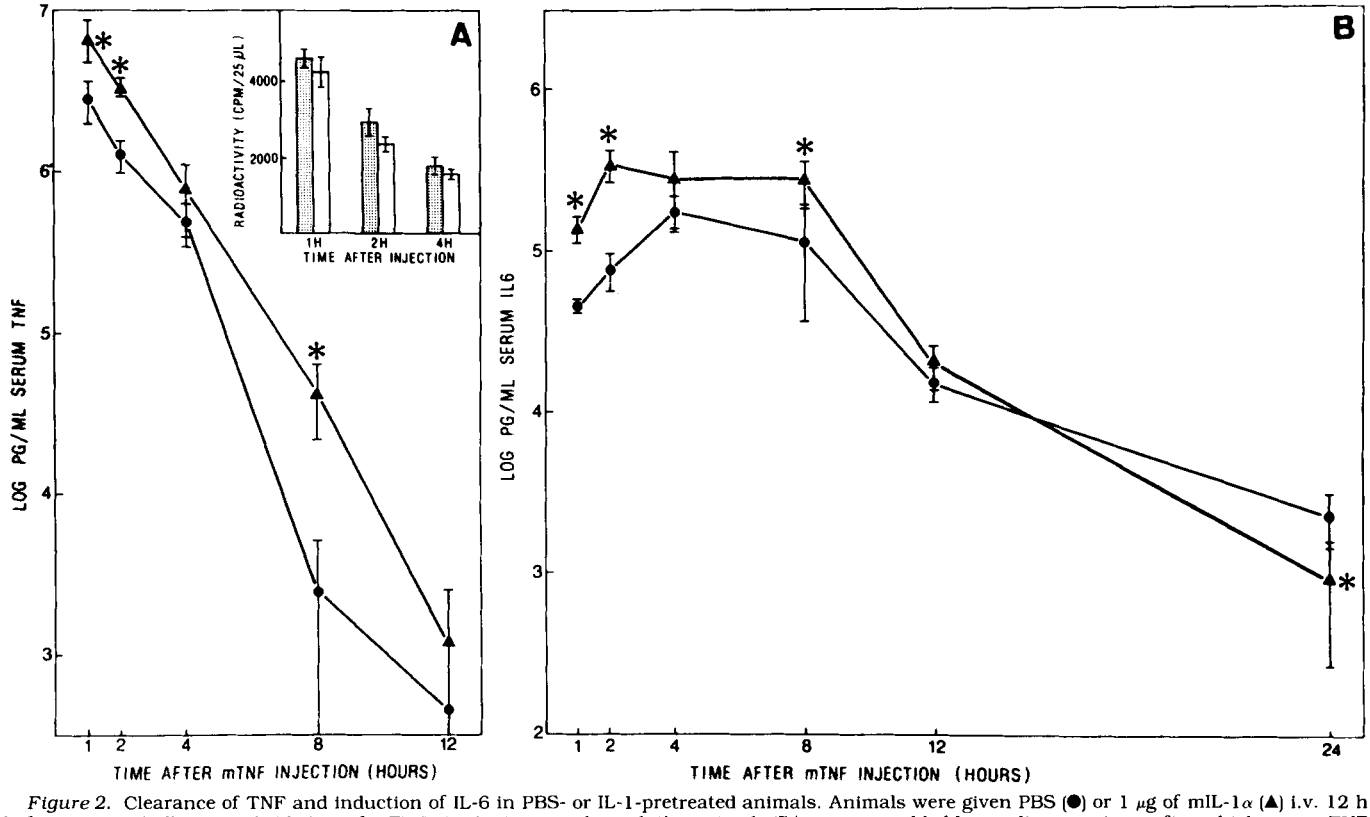

Figure 2. Clearance of TNF and induction of IL-6 in PBS- or IL-1-pretreated animals. Animals were given PBS (●) or 1 μg of mIL-1α (▲) i.v. 12 h before an i.v. challenge with 12.5 μg of mTNF. At the times indicated, the animals (5/group) were bled by cardiac puncture, after which serum TNF (*A*) and IL-6 (*B*) were determined as described in *Materials and Methods*. Significant differences ($p < 0.05$) according to the Student's *t*-test are indicated by an asterisk. *A* (inset), Radioactivity determined in serum aliquots derived from animals that had been given mTNF (10 μg) + [^{125}I]TNF (5×10^5 cpm/μg) at the times indicated. The mice were bled at the retro-ocular plexus and radioactivity was counted in 25-μl aliquots of serum ($n = 3$). Shaded bars show mIL-1α-pretreated, open bars PBS-pretreated animals (−12 h).

membranes prepared. After protein determinations and equalization of the amounts of membrane proteins used per mouse for the binding assay, [^{125}I]mTNF was allowed to bind for 4 h at 4°C with equal amounts of membrane proteins. As can be seen in Figure 3, the specific binding on membranes was identical for the two groups of animals. The mean amount of specific binding was 114 cpm and 114.5 cpm for PBS and IL-1-pretreated animals, respectively.

Tissue distribution of TNF in PBS- or IL-1-pretreated animals. As described in *Materials and Methods*, [^{125}I] mTNF was injected i.v. in PBS- or IL-1-pretreated mice. Organs were collected 30 min or 6 h after the injections. The radioactivity found in the different organs is represented in Figure 4*A* and 4*B*. No significant differences could be detected between both groups, neither at the early nor at the later time point. The late accumulation of radiolabel in the stomach is consistent with a previous report (17), in which evidence is provided that this organ accumulates released, free iodine, but not the TNF protein.

TABLE VI

GalN inhibits IL-1-induced protection against rmTNF + GalN lethality

Pretreatment[a]	Lethality[b]	
	12 h	72 h
PBS	14/15	15/15
2 μg IL-1	0/15	0/15
20 mg GalN	8/15	15/15
20 mg GalN + 2 μg IL-1	2/10	9/10
2 μg IL-1 + 20 mg GalN + 20 mg Urd	0/5	0/5
20 mg Urd	4/5	5/5
20 mg Urd + 20 mg GalN	2/5	5/5
2 μg IL-1 + 20 mg Urd	0/5	0/5

[a] Pretreatment was given i.p. (0.25 ml) 12 h before the challenge with 0.5 μg of rmRNF + 20 mg of GalN given i.p. (total volume: 0.5 ml).

[b] Number of dead mice vs total number of mice recorded 12 h and 72 h after the challenge injection (no further deaths occurred).

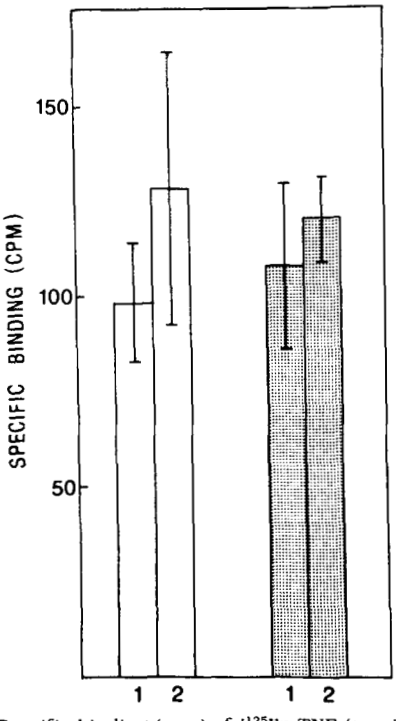

Figure 3. Specific binding (cpm) of [^{125}I]mTNF (specific activity: 7 × 10^7 cpm/μg) on membranes of hepatocytes obtained 12 h after injection of PBS (open bars) or of 2 μg of hIL-1β (shaded bars). The numbers 1 and 2 represent two different mice. The preparation of the membranes and the binding assay are described in *Materials and Methods*. Specific binding of [^{125}I]mTNF on membranes is defined as the difference between total binding of [^{125}I]mTNF (cpm) minus the binding of [^{125}I]mTNF in the presence of an excess of unlabeled mTNF (cpm).

DISCUSSION

Several lines of evidence indicate that host defense mechanisms and events leading to septic shock are mediated by an interplay of several small protein and non-protein molecules. In this respect, the cytokines TNF and IL-1 deserve special attention.

TNF can cause septic shock-like pathophysiologic changes in laboratory animals (18). Moreover, LPS-caused lethality was delayed or prevented by pretreating the animals with anti-TNF antibodies (2). Also, TNF levels were found to correlate with lethality in meningococcal infection (19) and in septic shock (3).

IL-1, another multifunctional cytokine, was found in the serum of septic shock patients (3), in the synovial fluid of rheumatoid arthritis patients (20), and in children suffering from purpurea (21). In animal models, IL-1 has been reported to be lethal after adrenalectomy (7) or in the presence of ActD or GalN (8). Regarding the latter results, however, we have never observed lethal effects of IL-1 in our C57BL/cnb mice, with or without GalN. It is possible that the difference of mouse strain or endotoxin contamination of the IL-1 preparation used could account for this discrepancy.

TNF and IL-1 share many biologic activities (22–24) and can induce each others' production, at least in vitro (25, 26). Furthermore, co-administration of IL-1 was found to augment TNF susceptibility of mice in a synergistic way (27, 28). In contrast, pretreatment with hIL-1α could protect mice against the lethality induced by hTNF or hIL-1α in combination with ActD or GalN (8). Protective properties of IL-1 were also observed against deleterious effects of *Candida albicans* (29), *E. coli*, *Klebsiella*, and *Pseudomonas* (30), whereas TNF showed protection against *Listeria*, Calmette-Guérin bacillus, and *Leishmania* (31).

We developed a model to study the mechanisms contributing to the lethality observed after TNF administration. As previously explained (14), only mTNF, but not hTNF (in doses up to several hundred micrograms per mouse), can cause lethality in C57BL/cnb mice. When TNF was given together with GalN, not only was a very strong sensitization for lethality seen, but the species specificity disappeared also. These observations indicate that at least two mechanisms are cooperating to produce the observed lethality: a "challenging" effect, shared by mTNF and hTNF, which only leads to lethality when a second sensitizing effect—for example, the inhibition of endogenous protective mechanisms—is present. This sensitizing effect can be exerted to a certain extent by mTNF, but not by hTNF, and can also be provided by other sensitizing molecules such as IL-1 or GalN, neither of which is lethal on its own. The sensitization of GalN for TNF is not due to an alteration in pharmacokinetics of TNF or IL-6[4]. GalN is a specific hepatotoxic sugar derivative: it causes a depletion of UTP in hepatocytes, leading to cessation of transcription and translation (32). The effect of GalN on LPS and TNF lethality is very drastic, but is fully reversible by administration of Urd, indicating that the GalN effect is indeed due to inhibition of macromolecular biosynthesis (33). For these and other reasons, the GalN model was accepted as being very suitable for the study of endotoxin mediators (34).

Some years ago, Freudenberg and Galanos (35) observed that animals treated with small amounts of LPS were refractory to the lethal effects of LPS/GalN. By using transfer experiments with macrophages, they were able to identify this cell type as a mediator in this LPS-induced desensitization (we were able to show that TNF and IL-1 do indeed protect against LPS/GalN-induced lethality).

Wallach et al. (8) observed that hIL-1α or hTNF, when given prior to a lethal combination of hTNF and ActD or GalN, prevented rapid mortality. They suggested that this protective effect was the result of an augmentation of antagonistic mechanisms that can be inhibited by blocking macromolecular synthesis. We have taken these observations as a basis for the studies described in this paper. In all experiments, however, we used mTNF instead of hTNF for challenging purposes.

Our results show the following: IL-1 protects against lethal combinations of hTNF/GalN, mTNF/GalN, and LPS/GalN. The four IL-1 species tested protect completely

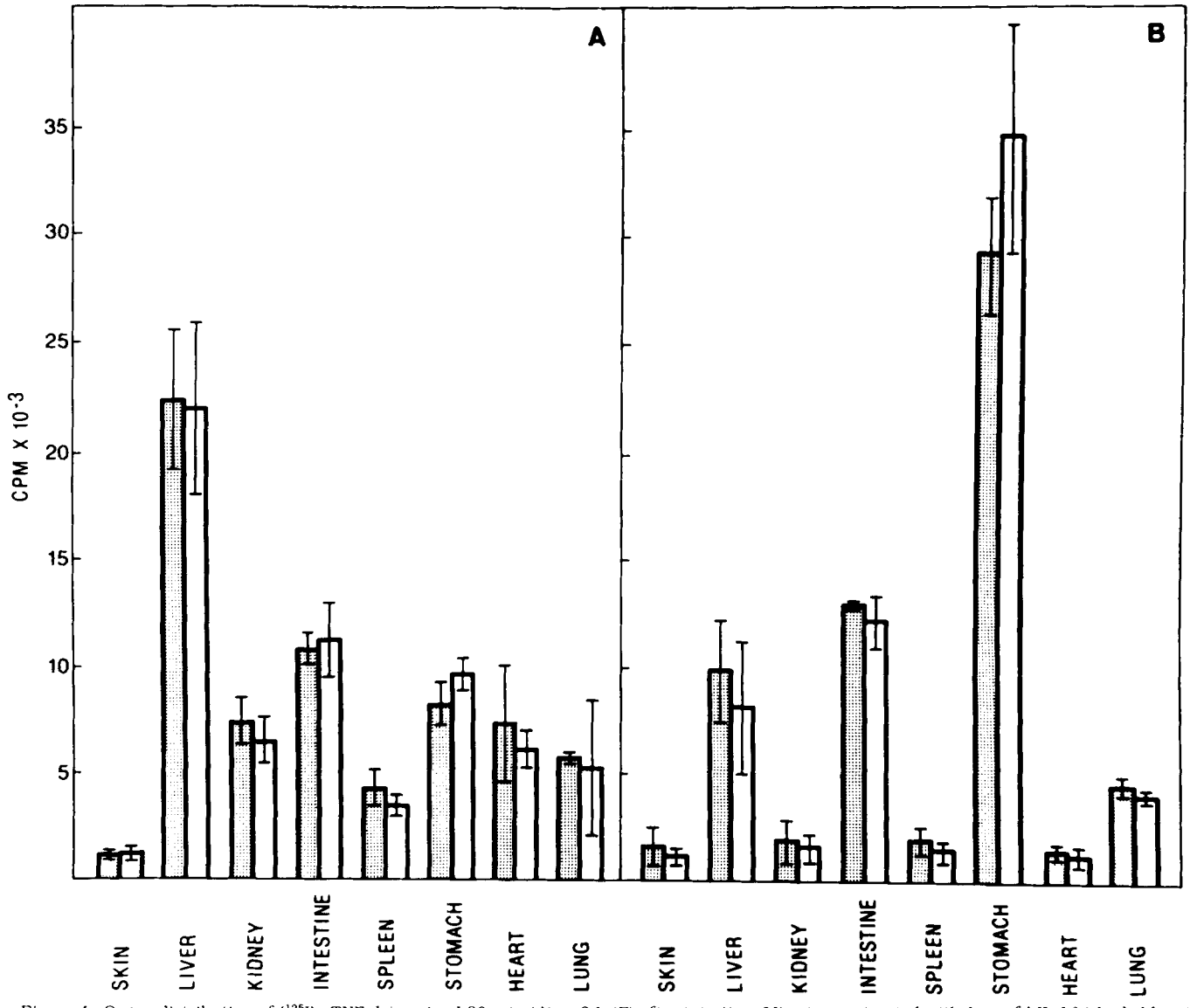

Figure 4. Organ distribution of [^{125}I]mTNF determined 30 min (*A*) or 6 h (*B*) after injection. Mice i.v.-pretreated with 1 μg of hIL-1β (shaded bars) or PBS (open bars) were i.v. injected with 5×10^5 cpm [^{125}I]mTNF + 10 μg of mTNF. Data are expressed as absolute radioactivity determined in each organ and recalculated to standard-sized organs as described in *Materials and Methods*.

against mTNF/GalN; no species specificity of IL-1 was noted. The IL-1 effect could still be observed with 0.1 μg of IL-1, but disappeared at lower concentrations. In kinetic studies, protection was achieved with IL-1 given between 4 and 20 h before the challenge; optimal protection seemed to have been obtained when IL-1 was given 12 h before the challenge. Both mTNF and hTNF protected against mTNF/GalN. This is a rare example of non-species-specific activity of TNF in the C57BL/cnb mouse model.

We also observed that LPS had a limited protective capacity and that mIFN-γ could delay, but not diminish, the lethality. Since IL-6 can be induced by IL-1, TNF, LPS, or IFN-γ (36, 37), we reasoned that IL-6 might mediate the observed protection. But neither mIL-6 nor hIL-6, administered at doses between 0.2 and 5 μg, were able to desensitize the animals when given as a single injection. Because of the relatively fast clearance of injected IL-6[4] and because IL-6 is induced by TNF or IL-1 within a time frame of approximately 2 h (11), we performed infusions of hIL-6 over a 4-h period at two doses. The IL-6-infused animals were not desensitized to TNF/GalN lethality. These observations are important in view of one of our other observations, namely that the protective effect is mediated by the liver, one of the main target organs for IL-6. The most important role for IL-6 in the liver is the regulation of the acute phase protein gene expression. It has been reported that combinations of IL-6 and DEX (16) or IL-6 and IL-1 (38) are most effective in inducing acute phase reactants. In our system, however, combining IL-6 and DEX or IL-1 did not result in desensitization to TNF/GalN lethality.

The protective effects of TNF or IL-1 could be mediated by glucocorticoids. On the basis of earlier experiments (data not shown) that indicated that both IL-6 and IL-1 induce similar circulating glucocorticoid hormone levels as measured 30 min after the injection, we believed that glucocorticoids are not involved (since IL-1 does, and IL-6 does not, protect). However, to check this conclusion, we injected DEX 12 h before TNF/GalN and at a dose which is effective in vivo (7), but no protection against TNF/GalN-induced lethality was observed. DEX has been

shown to protect against TNF or LPS lethality[4] (7), but only when given immediately before the challenge. Furthermore, no protection was observed with ACTH at a dose of 1 μg/g of body weight (15). Finally, RU486, a synthetic glucocorticoid receptor antagonist, did not inhibit the IL-1-induced desensitization (results not shown).

We further studied the mechanisms of IL-1-induced protection. We first excluded some obvious explanations. A first pitfall could be that the protection was raised against the activity of GalN, rather than against TNF or LPS (e.g., the induction of a GalN-clearing mechanism). In Table V we demonstrate, however, that IL-1 (in casu mIL-1α), when given 12 h before, completely prevented the animals from dying after a normally 100%-lethal injection of mTNF (without GalN). Although the IL-1-pretreated animals survived after the TNF challenge, they still showed the same signs of illness as control animals (e.g., a significant decrease in body temperature).

A second plausible explanation for an IL-1-induced protection could be that a TNF-clearing mechanism is activated by IL-1. Studies of TNF clearance, however, indicate the opposite: IL-1-pretreated animals at all time points after an mTNF challenge showed more TNF activity still present in circulation. So far, we have no satisfying explanation for this observation. One possibility could be that IL-1 treatment has a priming effect on cells producing endogenous TNF in response to TNF. Another explanation could be that pretreatment with IL-1 downregulates the receptors for TNF and by doing so prevents the effects of TNF. Therefore, binding assays with [^{125}I] TNF were performed on hepatocytes derived from IL-1 or PBS-treated animals. However, no significant difference between both groups was observed. Also when the tissue distribution of radiolabeled TNF was studied at two time points, no difference between both groups could be detected. Since the liver has been claimed to be the main TNF-clearing organ (17), only a diminished functioning of the TNF receptors in IL-1-pretreated animals could explain the augmented serum TNF levels. The results indicate that circulating TNF levels and lethal outcome do not always correlate. In this respect, it is worthwhile to note that the levels of circulating TNF in patients during phase I trials with TNF were as high as those seen in patients with a lethal outcome in septic shock (3, 39). We also demonstrated that the IL-1-treated animals contained more circulating IL-6 after a challenge. This induction of IL-6 proves that the challenging TNF in the IL-1-pretreated animals is not only present, but it has also retained at least some of its biologic effects.

Because the hepatotoxic GalN can sensitize mice drastically to the lethal effects of TNF, we questioned whether the protection induced by the pretreatment would not reside in the augmentation of the mRNA or protein level of certain enzymes or secreted proteins in the liver. To test this hypothesis, we inhibited such synthesis during the pretreatment by adding GalN to the desensitizing IL-1. In a controlled experiment, we observed that when the liver function was blocked with GalN, the protective effect of IL-1 decreased by 90%. After addition of Urd, the protection induced by IL-1 was no longer blocked by GalN, indicating that the relevant activity of GalN was indeed restricted to the depletion of UTP. These results indicate that the desensitization by IL-1 is induced in the liver. The fact that the liver seems to play a predominant role in this system is not so surprising. That the liver is important in counteracting the deleterious effects of TNF and LPS can also be derived from the observations that hepatectomized animals are much more prone to lethality induced by serum obtained from LPS-treated animals (40). The fact that IL-1 obviously not only restores, but also augments this liver protection system is new. Consequently, the macrophage-mediated protection induced by LPS against LPS/GalN lethality, described by Freudenberg and Galanos (35), might also be liver-borne. IL-1 induces a wide variety of proteins in hepatocytes, directly or indirectly (41). On the basis of the results presented here, we can conclude that the protective effect of the liver does not consist of an increased clearance of TNF activity either by increased activity of the clearing organs, or by binding or degrading the TNF in circulation. The question of which liver-made molecules are responsible for this antagonistic effect remains to be answered. Two categories of molecules attract particular attention. The first category is that of the classical detoxification enzymes: putative mediators in TNF-induced lethality (platelet-activating factor, leukotrienes, PG, or others) might be cleared in the liver. For the clearing of leukotrienes, the classical detoxification systems of the liver were shown to play an important role (glutathione and cytochrome P-450) (42). However, IFN decreases the activity of most of the glutathione transferases (43), whereas TNF, LPS, and IL-1 in vivo, and IL-1 in vitro decrease, the cytochrome P-450 activity (44). Also phenobarbital, a potent inducer of cytochrome P-450, could not protect against TNF lethality (results not shown). Secreted proteins form another class. Some of the acute phase proteins, e.g., protease inhibitors, are already suspected of having an antagonistic effect on TNF-induced phenomena (45). In this respect it is worth noting that, depending on the in vitro systems, IL-1 can induce the same acute phase proteins (46) or different ones than IL-6 can (47). Additional experiments will have to identify the presumably secreted protein(s) responsible for the protective activity induced by IL-1 pretreatment.

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