INFECTION AND IMMUNITY, Aug. 1991, p. 2609–2614
0019-9567/91/082609-06$02.00/0

Vol. 59, No. 8

Experimental Elimination of Tumor Necrosis Factor in Low-Dose Endotoxin Models Has Variable Effects on Survival

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Received 27 November 1990/Accepted 27 May 1991

Tumor necrosis factor alpha (TNF) is thought to play a major role in the pathogenesis of septic shock. Anti-TNF antibody was preadministered in low-dose endotoxin lethality models in which BALB/c mice were challenged with small amounts of lipopolysaccharide following their sensitization with either carrageenan (CAR) or D-galactosamine (D-GalN). Although the antibody virtually eliminated circulating TNF in both the CAR and the D-GalN models, only the D-GalN model mice were afforded survival, adding to a growing body of evidence that substances other than TNF play a key role in endotoxin-induced lethality. Further examination of sera from these mice showed a much greater elevation of interleukin-6 levels in the CAR-sensitized group than in the D-GalN-sensitized group.

Tumor necrosis factor alpha (TNF), a cytokine secreted by macrophages in response to invasive stimuli, is thought to be a principal mediator of the gram-negative bacterium-induced manifestations of septic shock (36, 38). High serum TNF levels are generally seen in the circulation 1 to 2 h after endotoxin assault (4, 26), and the presence of detectable amounts of TNF in serum has in some instances been an excellent predictor of subsequent host illness (19, 43). Animals infused with TNF display biochemical and physiologic abnormalities much like those seen in animals with gram-negative bacterium-induced sepsis (36, 39). Experimental models of septic shock have shown that antibodies against TNF, passively administered, protect animals from the lethal effects of endotoxin and overwhelming sepsis (5, 37).

In an effort to better understand endotoxin-associated lethality, our laboratory has employed two animal models in which mice are sensitized to the lethal effects of low-dose endotoxin by preadministration of either D-galactosamine (D-GalN) or carrageenan (CAR). D-GalN, a hepatotoxin, has been shown by Galanos et al. to potentiate the lethal effects of endotoxin up to 100,000-fold (17). CAR, thought to be cytotoxic for macrophages, is able to increase host sensitivity to endotoxin by 100- to more than 3,000-fold (3).

Functional macrophages appear to be a prerequisite for endotoxin-induced lethality (10, 16), presumably in part because of their ability to secrete TNF. We had previously noted that when preadministered to experimental mouse models, silica particles, which are known to be toxic to macrophages (2), and casein, a milk solid capable of inhibiting normal macrophage function (24), enhanced survival of mice pretreated with D-GalN plus lipopolysaccharide (LPS) but not with CAR plus LPS. Levels of TNF in serum, however, were substantially reduced in both models (unpublished observations). We have therefore endeavored to examine the ability of anti-TNF antibody to elicit similar effects. Preadministration of anti-TNF in these same models did indeed offer protection to the D-GalN-plus-LPS-challenged mice but not to the CAR-plus-LPS-challenged mice, and yet levels of TNF in serum were virtually eliminated in both models. These data suggest the involvement of a critical element other than, or in addition to, TNF in CAR-plus-LPS-induced lethality. Upon further examination of sera from these animals, we found that CAR-sensitized mice produced significantly more interleukin-6 (IL-6) than their D-GalN-sensitized or saline control counterparts.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from Charles River Laboratories, Inc., Wilmington, Mass. Mice were 8 to 12 weeks of age at the time of use and were housed and cared for according to current guidelines established by the Animal Welfare Act. The experiments described in this study were conducted according to principles set forth in reference 21a. All experiments described utilized groups of 6 to 10 mice and were repeated a minimum of three times.

Low-dose endotoxin models. Groups of mice were injected intraperitoneally (i.p.) with 10 mg of D-GalN (purchased from Sigma, St. Louis, Mo.) dissolved in saline immediately prior to use, 1 mg of lambda CAR (Sigma) dissolved at a concentration of 5 mg/ml at 56°C in saline, or an equivalent volume of normal saline only. One hour later, mice were given 2 μg of LPS i.p. (purified *Escherichia coli* O111:B4 dissolved in saline, obtained from Calbiochem-Behring Diagnostics, La Jolla, Calif.). Mice were lightly anesthetized with inhalant Metofane (Pitman-Moore, Inc., Washington Crossing, N.J.) and bled via retro-orbital sinus puncture at 1, 2.5, 4, and 6 h after LPS injection, and their sera were harvested for TNF assay. Animals were observed for 48 h, and their survival times were recorded. Some of the animals were bled at 1, 3, and 6 h after LPS injection for assessment of levels of IL-6 in serum.

Anti-TNF antibody administration. Groups of mice were injected i.p. with up to 4.0×10^5 U of rabbit anti-mouse TNF (neutralizing activity of 1×10^6 U/ml; Genzyme, Boston, Mass.) or with an equivalent volume of normal rabbit serum (NRS) 5 h prior to challenge with sensitizing agents plus LPS. Serum samples were obtained at 1 and 3 h post LPS injection, and survival times were recorded. To check reagent specificity, in vitro blocking experiments were done whereby anti-TNF serum or NRS was incubated with TNF-positive mouse serum, which was subsequently assayed for residual TNF. Activity of TNF in serum was completely

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inhibited by preincubation with anti-TNF serum but not with NRS (data not shown).

Tolerance induction. Mice were injected i.p. on days −7, −5, −3, and −1 with either 2 μg of LPS or an equivalent volume of saline. On day 0, they received CAR plus LPS, D-GalN plus LPS, or saline plus LPS in the manner previously described. Mice were bled and monitored as noted above.

TNF assay. Concentrations of TNF in serum were measured by the following cytotoxicity assay described by Flick and Gifford (12). Dilutions of individual experimental mouse sera were made in RPMI 1640 (GIBCO, Grand Island, N.Y.) containing 10% fetal calf serum (GIBCO) and were added in quintuplicate to flat-bottom 96-well microtiter plates (Becton Dickinson, Oxnard, Calif.) which had been seeded the previous day with 5×10^4 L929 cells per well and incubated at 37°C. Half-log dilutions of recombinant TNF (rTNF) (activity = 4×10^7 U/mg; Genzyme), ranging from 1,000 to 0.1 pg per culture, were added to some wells to provide a known standard. At this time, 1 μg of actinomycin-D (Sigma) was also added to each well. Plates were washed the following day, stained with crystal violet (Gram stain formulation; Difco, Detroit, Mich.), and read on a Dynatech MR 600 Microplate Reader at a wavelength of 596 nm. Experimental concentrations of TNF in serum were extrapolated from a standard curve constructed from the known TNF dilutions.

IL-6 assay. Levels of IL-6 in serum were measured by its ability to cause proliferation of the murine B-cell hybridoma line B9 (1). Twofold dilutions of serum samples in RPMI supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol (Sigma), and 50 μg of gentamicin (GIBCO) per ml were added to 96-well flat-bottom plates containing 10^3 B9 cells per well. Plates were incubated for 76 h, pulsed with 0.4 μCi of [^{3}H]thymidine (specific activity = 6.7 Ci/mM; New England Nuclear, North Billerica, Mass.), and assessed for incorporated radioactivity. Dilutions of samples needed to obtain half-maximal proliferation were related to a standard curve, and IL-6 titers were expressed in nanograms per milliliter.

In vivo sensitivity to rTNF. Mice received 10 mg of D-GalN, 1 mg of CAR, or 0.2 ml of saline i.p. One hour later, they were given between 4 and 400 ng of rTNF either i.p. or intravenously. Deaths were reported over a 48-h period.

Statistical analysis. All data presented are expressed as the mean ± the standard error of the mean. Statistical analysis of TNF and IL-6 values was performed by using the Student *t* test, and survival data were assessed by chi-square analysis. Graphs were derived from single representative experiments.

RESULTS

Effects of CAR, D-GalN, and/or LPS on TNF levels and survival. The doses of CAR (1 mg) or of D-GalN (10 mg) plus LPS (2 μg) were chosen because they were found to be the smallest amounts of these reagents that would consistently produce 100% lethality in these mice. Utilization of minimal quantities of these substances was thought to limit their sensitizing capabilities so that any protective effect that the anti-TNF antibody produced might be seen more easily. Neither CAR, D-GalN, nor LPS alone at these doses affected survival. TNF could not be detected in sera of mice which received CAR or D-GalN alone, while mice receiving LPS alone produced moderate quantities of TNF. Mice sensitized with CAR or D-GalN prior to receiving LPS did show somewhat elevated levels of TNF compared with those of mice receiving LPS alone at the earliest time point. However, neither CAR nor D-GalN, when used in combination with LPS, caused TNF levels to remain high beyond the normal time course generally seen with endotoxin-challenged animals (Fig. 1).

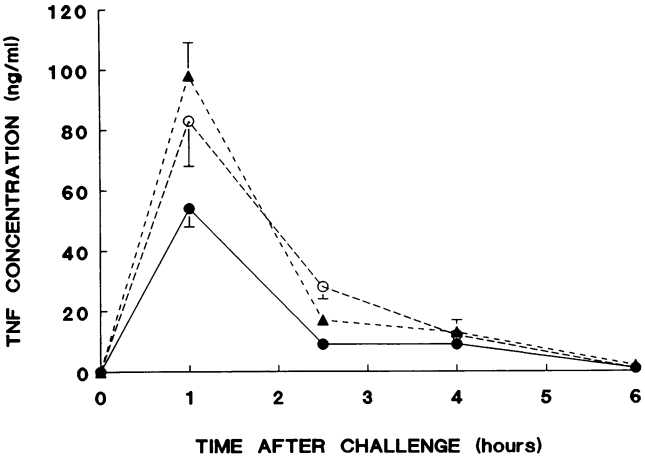

FIG. 1. Kinetics of TNF production in mice challenged with sensitizing agents and endotoxin. Mice were injected with D-GalN (10 mg), CAR (1 mg), or saline and 1 h later with LPS (2 μg) or saline (*n* = eight mice per group). Serum samples obtained at 1, 2.5, 4, and 6 h post LPS injection were assayed for TNF. Survival rates were 0% for mice receiving D-GalN plus LPS, 5% for mice receiving CAR plus LPS, and 100% for mice receiving saline plus LPS, D-GalN plus saline, or CAR plus saline. Mice receiving either D-GalN plus saline or CAR plus saline did not produce detectable quantities of circulating TNF. Symbols: ●, saline plus LPS; ○, CAR plus LPS; ▲, D-GalN plus LPS.

Effects of CAR, D-GalN, and/or LPS on IL-6 levels. Sera from mice which were sensitized with D-GalN or CAR and challenged with LPS were tested for the presence of IL-6 at 1, 3, and 6 h post LPS administration. Although all groups of mice produced modest quantities of IL-6 at the 1-h time point, levels of this cytokine were markedly increased at the 3- and 6-h time points in mice sensitized with CAR ($P < 0.001$). Mice given D-GalN or saline, plus LPS, produced quantities of IL-6 at 3 and 6 h that were either similar to or slightly higher than 1-h levels (Fig. 2). Animals which received saline or CAR alone did not elaborate significant amounts of IL-6, while mice injected with D-GalN alone did display a slight elevation of this cytokine.

Pretreatment with anti-TNF antibody. Some groups of animals received polyclonal rabbit anti-TNF antibody or NRS 5 h before being challenged with D-GalN, CAR, or saline, plus LPS. Such antibody pretreatment ablated TNF levels in serum in all groups (Fig. 3). However, pretreatment with antibody against TNF allowed 100% of the D-GalN-plus-LPS-challenged mice to survive (versus 0% of the controls), while allowing only 10% of the CAR-plus-LPS-challenged mice to survive (versus 0% of controls). Survival differences between the anti-TNF-pretreated D-GalN and CAR models were highly significant ($P < 0.001$).

Sera from identically treated animals were also assessed for IL-6 levels (Table 1). Anti-TNF pretreatment of mice which received either D-GalN or saline, plus LPS, partially suppressed IL-6 levels in serum at the 1-h time point ($P = 0.06$ for mice in the D-GalN-plus-LPS group; $P = 0.01$ for mice in the saline-plus-LPS group). Anti-TNF pretreatment

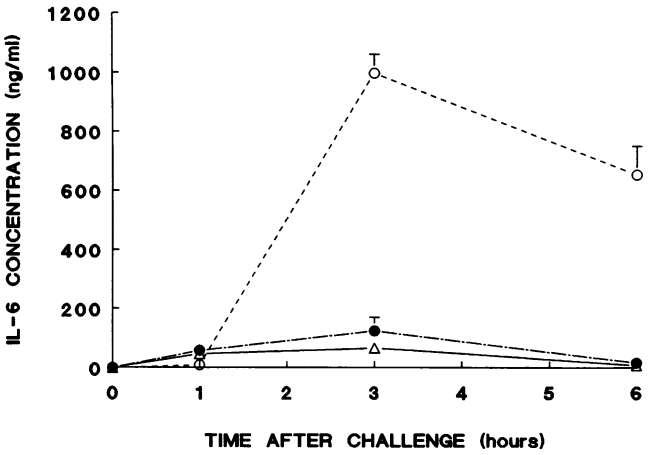

FIG. 2. Kinetics of IL-6 production in mice challenged with sensitizing agents and endotoxin. Mice received D-GalN (10 mg), CAR (1 mg), or saline and LPS (2 μg) or saline 1 h later (n = six mice per group). Mice were bled at 1, 3, and 6 h post LPS injection, and IL-6 levels in serum were measured. Animals given either CAR plus saline or saline alone did not produce significant quantities of IL-6, while those given D-GalN plus saline produced a very slight amount. Symbols: △, saline plus LPS; ○, CAR plus LPS; ●, D-GalN plus LPS.

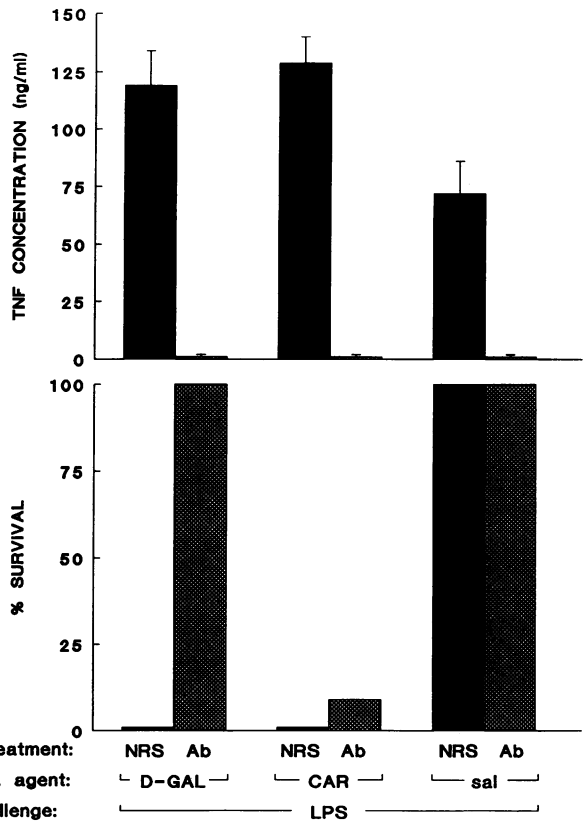

FIG. 3. Effects of anti-TNF pretreatment on TNF levels in serum and survival. Mice were injected with 4 × 10^5 U of rabbit anti-mouse TNF (Ab) or NRS at −5 h. D-GalN (10 mg), CAR (1 mg), or saline was given at time zero, followed by LPS (2 μg) 1 h later (n = 10 mice per group). Serum samples were obtained 1 h post LPS injection and were assessed for TNF levels. Mice were observed for 48 h, and deaths were recorded. Animals that received anti-TNF or NRS plus either sensitizing agent plus saline did not produce detectable TNF levels, nor was their survival affected.

did not affect the IL-6 levels in sera of mice which had been challenged with CAR plus LPS at the 1-h time point. At the 3-h time point, antibody pretreatment significantly inhibited IL-6 production in mice injected with either D-GalN plus LPS (P = 0.005) or saline plus LPS (P < 0.001). However, mice pretreated with anti-TNF antibody prior to receiving CAR plus LPS demonstrated only a marginally significant decrease in IL-6 levels in serum (P = 0.11) at the 3-h time point.

Effects of inducing tolerance with LPS. Pretreatment with small amounts of LPS has been previously reported to induce tolerance to D-GalN–LPS-triggered lethality (15). We therefore performed similar experiments utilizing both the D-GalN and CAR models in order to determine whether inducing tolerance with LPS would affect survival and/or TNF levels in serum in the two models differently. TNF levels in serum in groups of mice were measured following induction of tolerance with LPS and subsequent treatment with D-GalN, CAR, or saline, plus LPS. All tolerance-induced groups showed significantly depressed TNF concentrations in serum after the standard challenge regimens (Fig. 4). In this set of experiments, 100% of the tolerance-induced mice in all groups survived, whereas none of the sensitized non-tolerance-induced controls survived.

Sensitivity of mice to rTNF. To determine whether mice receiving either CAR or D-GalN alone might be especially sensitive to TNF, animals were given saline, CAR, or D-GalN, followed by amounts of rTNF ranging from 4 to 400 ng. Somewhat surprisingly, only the mice receiving D-GalN were unable to tolerate the largest rTNF challenge. All CAR-sensitized and saline control mice survived a 400-ng rTNF challenge, whether given i.p. or intravenously. For D-GalN-sensitized mice, the 50% lethal dose of rTNF was 40 ng (data not shown).

DISCUSSION

TNF is thought to be the proximal mediator in a cascade of cytokine events which lead to the pathophysiological effects observed in the septic host. Although other cytokines, such as IL-1, IL-6, and lymphotoxin (TNFβ), have been shown to cause many of the same physiologic abnormalities as TNF (25, 30, 44) or even to enhance the effects of TNF (42), examples in which TNF itself is absent in such animals are few. In our laboratory, we have developed a pair of low-dose endotoxin lethality models in which CAR- or D-GalN-sensitized, LPS-challenged mice were pretreated with anti-TNF antibody. Although TNF levels in serum were dramatically reduced or eliminated in both models, the animals which had been sensitized with CAR failed to survive an LPS challenge. D-GalN-sensitized mice were completely protected by antibody pretreatment. Clearly, different mechanisms are at work in the two models.

In contrast to the above results was the finding that all LPS tolerance-induced mice, whether subjected to the CAR-plus-LPS protocol or to the D-GalN-plus-LPS protocol, not only showed the expected reduction of circulating TNF levels but demonstrated 100% survival rates. Tolerance induction with LPS is apparently able to overcome the sensitizing properties of CAR and/or the toxicity of LPS in a way that anti-TNF antibody is unable to do, suggesting that, unlike LPS tolerance induction regimens, this antibody does not exert its influence by rendering the mice unresponsive to challenge.

TABLE 1. Effects of anti-TNF antibody administration on IL-6 levels in sera of sensitized and LPS-challenged mice

Pretreatment	Treatment	IL-6 level (ng/ml) ± SEM (% dec.)[a] at time postinjection	
		1 h	3 h
NRS	D-GalN + LPS	50.7 ± 9.2	193.9 ± 32.8
Anti-TNF	D-GalN + LPS	32.2 ± 1.3 (37%)	52.2 ± 1.4 (73%)
NRS	CAR + LPS	24.8 ± 1.5	723.2 ± 124.2
Anti-TNF	CAR + LPS	24.4 ± 2.0 (2%)	479.8 ± 86.8 (34%)
NRS	Saline + LPS	48.0 ± 6.0	61.0 ± 3.7
Anti-TNF	Saline + LPS	28.3 ± 1.7 (41%)	11.0 ± 1.4 (82%)
	D-GalN	0.57	ND[b]
	CAR	0.04	ND
	Saline	0.02	ND

[a] Percent decrease (dec.) of IL-6 in serum observed with anti-TNF antibody-pretreated mice compared with that of NRS-pretreated mice in each grouping.
[b] ND, Not determined.

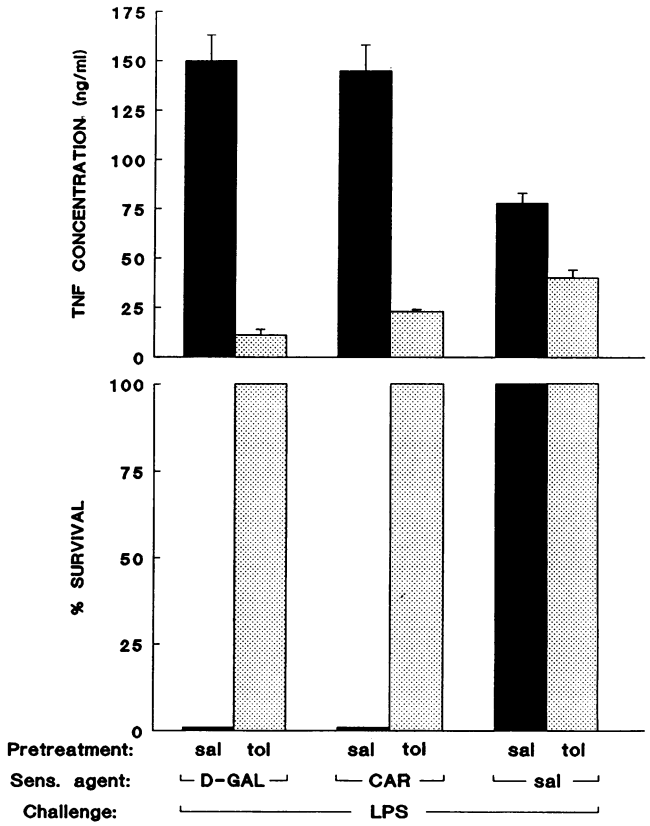

FIG. 4. TNF concentration in serum and survival in LPS tolerance-induced (tol) mice. Mice received either LPS (2 μg) or saline (sal) on days −7, −5, −3, and −1. Animals were then given D-GalN (10 mg), CAR (1 mg), or saline at time zero, followed by LPS (2 μg) or saline 1 h later (n = six mice per group). Serum samples were obtained 1 h post LPS injection and were assessed for TNF levels. TNF could not be detected in sera of mice receiving LPS for tolerance induction plus either sensitizing agent plus saline. Animals were observed for 48 h, and their survival times were noted.

It is also apparent from these studies that mice which received sublethal doses of LPS alone elaborated very significant amounts of TNF. The amount of TNF produced in sera of mice receiving only LPS was nearly 100 times the amount produced in sera of mice treated with anti-TNF plus sensitizing agents plus LPS. However, those mice which were given LPS alone all survived despite their elevated TNF levels in serum, while many of the experimentally TNF-suppressed groups died. These findings correlate nicely with a report by Feuerstein et al. that showed that a substantial discrepancy between TNF elaboration and mortality rate can exist. They noted that, in rats, the doses of LPS needed to produce 50% mortality were 350-fold higher than the doses necessary to produce 50% of the maximum obtainable TNF levels in serum (11). Furthermore, when they infused rats with enough human TNF to produce levels of TNF in plasma many times higher than those elicited by a lethal dose of LPS, survival was unaffected.

The reason that the CAR model differs from the D-GalN model regarding the relationship of TNF levels to lethality is not entirely clear. The sensitizing properties of D-GalN, a hepatotoxin, are of short duration (approximately 3 h) and are apparently associated with decreasing UTP levels, which in turn impair biosynthesis. These fleeting effects are confined to the liver and correspond to the time when biochemical alterations are in process, not when cell injury or cell death has occurred (17). Freudenberg et al. have further demonstrated that the sensitizing effects of D-GalN are most likely due to a temporary lowering of the threshold of susceptibility in the host to the toxic products of macrophages rather than an enhancement of the actual mechanisms of endotoxicity (16).

CAR, on the other hand, has multiple effects on the whole organism. In addition to being cytotoxic to macrophages, probably via destabilization of the lysozomal membrane (2, 3, 8), CAR has been shown to inhibit complement 1 activity (6), has a dual, dose-dependent effect on clotting time (32), suppresses delayed hypersensitivity-type skin reactions (31), suppresses gamma interferon production (35), induces IL-1 production (29), and induces prolonged suppression of anti-sheep erythrocyte responses via Thy 1^+ Lyt 2^+ cells (41). Unlike D-GalN, it can increase the sensitivity of mice to the effects of endotoxin when given up to 24 h before (28) or as long as 8 h after (unpublished observations) challenge with LPS. The broad range of effects of CAR makes it difficult to determine to what extent attendant mortality is due to CAR-induced endotoxin sensitivity and how much of it is due to possibly unrelated but added effects of CAR itself.

IL-6, another cytokine implicated in the pathophysiology of endotoxemia, is thought to be secreted by, and to directly affect, a great variety of cell types (for a review, see reference 40) and to be largely responsible for the induction of the hepatic acute-phase response (18). Its production can be triggered directly by endotoxin (20, 23), by viral infection (9, 27), or by the presence of IL-1 (33) or TNF (7, 22), among other factors. Regulation of IL-6 production appears to be complex and is often but not always linked to circulating TNF levels. Zabel et al. have recently shown that treatment of endotoxin-challenged human subjects with pentoxifylline, a drug able to protect various animal models from the lethal effects of LPS, caused selective inhibition of TNF without affecting IL-6 levels (45). However, other studies performed by Fong et al. demonstrated that passive immunization of baboons with anti-TNF prior to challenge with *E. coli* not only eliminated TNF but dramatically reduced circulating levels of both IL-1 and IL-6 (14).

There is some question as to whether IL-6 by itself demonstrates an enhancement (34) or a relative lack of toxicity (7, 40) with regard to septic shock. Assessment of IL-6 levels in CAR- or D-GalN-sensitized, LPS-challenged mice showed that very high levels of this cytokine continued to circulate in CAR-sensitized mice (but not in D-GalN-sensitized mice) even if they had been pretreated with anti-TNF antibody. While the appearance of significant quantities of IL-6 in the serum of CAR-sensitized animals does not indicate that this cytokine is the cause of the high mortality rate observed in this group, it does support the findings of others (13, 21) that its presence in large amounts is very often associated with endotoxemic shock. A recent study by Starnes et al. demonstrated that mice pretreated with anti-IL-6 monoclonal antibody prior to challenge with a lethal dose of *E. coli* were protected from death, suggesting that IL-6, either by itself or through the induction of other factors, may be partially responsible for the pathophysiology of sepsis (34). We plan to examine IL-1 levels in these animals in the near future, since this factor is also thought to figure prominently in the consequences of endotoxemia (25, 42), and especially in light of the fact that Okuda et al. have reported that CAR is able to stimulate IL-1 production in murine macrophages (29).

We also considered the possibility that the high mortality observed with mice receiving CAR or D-GalN was due to a hypersensitivity induced by these compounds to TNF itself. Mice given D-GalN were in fact more than 10 times as sensitive to injected rTNF as saline controls, whereas mice given CAR were not affected by the dosages used. However, since D-GalN-treated mice, which were more sensitive to rTNF, could be protected by anti-TNF administration, and since the CAR-treated mice, which seemed relatively unaffected by rTNF injection, could not be protected by anti-TNF antibody, we concluded that death of the CAR-sensitized group could not be explained simply by hypersensitivity to levels of TNF that were possibly too low to be detected in a standard assay.

In conclusion, the results of these studies show that there are clear differences in the ways CAR and D-GalN sensitize mice to the effects of endotoxin administration. Experimental elimination of TNF did not afford survival to CAR-sensitized mice, and IL-6 levels were dramatically elevated in this group. Additional studies will be needed to shed light on the possible involvement and interplay of other factors in these animals.

ACKNOWLEDGMENTS

This work was supported by Naval Medical Research and Development Command Work Unit No. MR041.20.005-1004.

We thank Florence Rollwagen, John Ryan, and Thomas McKenna for their thoughtful suggestions and their generous donations of mice and reagents.

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