INFECTION AND IMMUNITY, June 1991, p. 2110–2115
0019-9567/91/062110-06$02.00/0

Vol. 59, No. 6

Tumor Necrosis Factor Alpha Mediates Lethal Activity of Killed Gram-Negative and Gram-Positive Bacteria in D-Galactosamine-Treated Mice

M. A. FREUDENBERG AND C. GALANOS*

Max-Planck-Institut für Immunbiologie, 7800 Freiburg, Federal Republic of Germany

Received 13 September 1990/Accepted 28 February 1991

Treatment with D-galactosamine increases sensitivity of lipopolysaccharide (LPS)-responder mice to the lethal effects of LPS, while nonresponder mice remain resistant (M. A. Freudenberg, D. Keppler, and C. Galanos, Infect. Immun. 51:891–895, 1986). In the present study it is shown that, in contrast to LPS, killed gram-negative bacteria (*Salmonella abortus equi* and *S. typhimurium*) were highly toxic for D-galactosamine-treated LPS-responder (C57BL/10 ScSN and C3H/HeN) and -nonresponder (C57BL/10 ScCR and C3H/HeJ) mice, although to a higher extent in the former strains. Also, killed gram-positive bacteria (*Staphylococcus aureus*, *Propionibacterium acnes*, and *Mycobacterium phlei*) exhibited toxicity for D-galactosamine-treated mice, LPS-responder and -nonresponder mice being equally susceptible. Evidently, bacterial components other than LPS may exhibit lethal effects in sensitized animals. In all cases, the lethality of LPS and of bacteria was inhibited by anti-tumor necrosis factor alpha (TNF-α) serum. While LPS induced TNF-α in vitro only in macrophages from LPS-responder mice, gram-negative and gram-positive bacteria induced TNF-α also in macrophages from LPS-nonresponder mice. The data show that TNF-α is a common endogenous mediator of the lethal activity of gram-negative and gram-positive bacteria.

The mechanisms of septic shock have been the subject of intensive investigation for several decades. In gram-negative microorganisms, endotoxins (lipopolysaccharides [LPS]) constitute the main pathogenetic factor. Purified LPS induces a spectrum of pathophysiological activities, many of which are strikingly similar to those seen in patients with gram-negative infections. Gram-positive bacteria do not synthesize endotoxin, yet the clinical manifestations of shock induced by gram-negative and gram-positive bacteria are in many respects indistinguishable (33), suggesting that similar host-dependent mechanisms may underly their development.

Today there exists general agreement that the lethal activity of LPS is induced via endogenous mediators formed on interaction of LPS with cellular targets. Macrophages were shown to mediate the lethal action of endotoxin (11, 26), and tumor necrosis factor alpha (TNF-α) was recognized as an important mediator implicated in the development of endotoxicity (4, 25). TNF-α was originally discovered as an LPS-induced macrophage product with tumor-necrotizing (in vivo) and tumoricidal (in vitro) properties. The importance of TNF-α in endotoxicity was first recognized by Beutler et al. when they showed that passive immunization of mice against TNF-α afforded a significant protection against subsequent challenge with LPS (5).

The lethal activity of endotoxin may be enhanced under different experimental conditions. Infection of mice with gram-negative microorganisms (*Salmonella typhimurium* and *Coxiella burnetii*) leads to a dramatic increase of their sensitivity to LPS (6, 12, 21, 27). Also, a number of gram-positive bacteria (*Mycobacterium bovis* bacillus Calmette-Guérin and *Propionibacterium acnes*) enhance the sensitivity of the treated animals to LPS when administered to mice (reviewed in reference 6). In all cases investigated so far, the sensitization to LPS induced by different bacteria was paralleled by a sensitization to the lethal activity of TNF-α (13, 21). Sensitization to endotoxin may also be induced by a number of hepatotoxic agents such as lead acetate (28), α-amanitin (29), or D-galactosamine (14). Mice treated with D-galactosamine prove a useful model for studying the initial events in the induction of endotoxin lethality. Mice thus treated become approximately 100,000 times more sensitive to the lethal effects of LPS (50% lethal dose [LD_{50}], 1 to 10 ng). However, this is true only for mice which are genetically responsive to LPS. LPS-nonresponder C3H/HeJ mice remain resistant to the lethal effects of LPS also after D-galactosamine treatment (LD_{50}, 1.8 mg) (11). The unresponsiveness of LPS-resistant mice was shown to be due to the absence of LPS-responsive macrophages. D-Galactosamine-treated LPS-nonresponder mice, transferred with LPS-responder macrophages, became sensitive to submicrogram amounts of LPS (11). It was shown recently that D-galactosamine sensitizes to LPS by increasing susceptibility to the activity of the LPS-induced macrophage mediator(s) (10). Recombinant TNF-α was found to exhibit enhanced toxicity in D-galactosamine-sensitized mice, suggesting that sensitization by D-galactosamine may be directed towards the activity of this mediator. In contrast to LPS, TNF-α exhibits lethal effects also in LPS-nonresponder mice (18).

In the present study, the toxic properties of LPS and of a number of killed gram-negative and -positive bacteria were investigated in D-galactosamine-treated mice. In contrast to LPS, which was toxic only for LPS-responder mice, all bacteria used (gram negative and gram positive) exhibited lethal activity in responder as well as nonresponder mice. Evidence will be presented that the lethal activity of LPS for responder mice and that of the bacteria for responder and nonresponder mice were mediated by TNF-α. The results suggest that induction of TNF-α may be a common mechanism by which gram-negative and gram-positive bacteria induce septic shock.

* Corresponding author.

MATERIALS AND METHODS

Mice. LPS-responder mouse strains C57BL/10 ScSN and C3H/HeN of both sexes and their congenic LPS-nonresponder counterparts, C57BL/10 ScCR and C3H/HeJ (1, 7, 24, 30), were obtained from the breeding stock of our institute. For lethality tests, mice 10 to 20 weeks old were used. Six-week-old mice served as donors of bone marrow cells for macrophage cultures.

Lethality test. Lethal toxicity tests were carried out in D-galactosamine-sensitized mice (14). Unless otherwise stated, materials under test were injected intravenously into the lateral tail vein as a mixture with D-galactosamine (20 mg/mouse) in 0.2 ml of pyrogen-free phosphate-buffered saline (PBS). Deaths were recorded up to 24 h after injection.

Materials. *Salmonella abortus equi*, *Salmonella typhimurium* (C5), and *Staphylococcus aureus* were obtained from overnight cultures. They were washed twice with pyrogen-free PBS, pH 7.2, and cells were killed by heating in PBS at 100°C for 30 min. *Mycobacterium phlei* was grown in casein-peptone-yeast extract medium containing 1% glucose at 37°C for 36 h. *Propionibacterium acnes* ATCC 12930 was grown in cooked-meat medium (Difco Laboratories, Detroit, Mich.) at 37°C for 4 days without aeration. Harvested bacterial cells were washed as described above and killed at 65°C for 1 h. All heat-killed bacteria were centrifuged, washed twice with pyrogen-free distilled water, and lyophilized. For use, bacteria were suspended in pyrogen-free PBS. Endotoxin contamination in the gram-positive bacterial preparations was <0.1 pg/μg as determined by *Limulus* amoebocyte lysate test (31).

LPS of *S. abortus equi* was obtained from parent bacteria by the phenol-water method (34). It was purified further by the phenol-chloroform-petroleum ether procedure and converted to uniform triethylamine salt (15). It was stored as a sterile aqueous stock solution (10 mg/ml) at 4°C.

D-Galactosamine hydrochloride (Hepasamine) was purchased from C. Roth, Karlsruhe, Federal Republic of Germany. Actinomycin D-mannitol was purchased from Sigma Chemical Co., St. Louis, Mo. Rabbit anti-mouse TNF-α was purchased from Genzyme, Boston, Mass. Murine recombinant TNF-α was a kind gift from G. R. Adolf, Bender and Co., Vienna, Austria.

Macrophages. Macrophages were cultured from bone marrow precursors of the various mouse strains in the presence of L-cell condition medium, as described earlier (11). Cells obtained after 10 days of culture were centrifuged, washed twice with serum-free culture medium (high-glucose formulation of Dulbecco modified Eagle medium), and used immediately. At least 96% of the cells thus obtained were macrophages as determined by adherence, staining with α-naphthylbutyrate (nonspecific esterases), Pappenheim staining (morphology), and uptake of colloidal carbon (11).

Stimulation of TNF-α in vitro. Macrophages, 5×10^5, suspended in 1 ml of Dulbecco modified Eagle medium supplemented with 5% fetal calf serum (GIBCO Ltd., Paisley, Scotland) were placed in culture wells (24-well multidish, Nunclon; Nunc, Roskilde, Denmark). The substance under test (20 μl) was added, and the cells were incubated at 37°C for different time periods (30 min to 24 h) in a humidified atmosphere containing 8% CO_2. Cell-free supernatants collected for TNF assay were stored frozen at −70°C until use.

Measurement of TNF activity. TNF-α activity in macrophage supernatants was determined by a cytotoxicity assay, using TNF-sensitive (2) and TNF-resistant L-929 cells kindly provided by L. J. Old, Sloan-Kettering Institute, New York, N.Y. TNF-α activity is expressed as units per milliliter, 1 U being the amount of TNF-α causing 50% lysis of L-929 cells. This amount corresponds to 4.17 pg of a murine recombinant TNF-α used as the standard.

TABLE 1. Lethal toxicity of LPS and whole heat-killed bacteria in D-galactosamine-treated LPS-responder (C57BL/10 ScSN) and LPS-nonresponder (C57BL/10 ScSR) mice[a]

Agent	Amt (μg)	Lethality (%) in: C57BL/10 ScSN	Lethality (%) in: C57BL/10 ScCR
LPS			
S. abortus equi	0.0001	0	NT[b]
	0.001	20	NT
	0.01	100	0
	500	NT	0
Bacteria			
S. abortus equi	0.1	0	NT
	1.0	100	0
	10	100	20
	100	NT	100
S. typhimurium	0.1	0	NT
	1.0	100	0
	10	100	30
	100	30	100
Staphylococcus aureus	1	0	0
	10	40	60
	100	100	100
P. acnes	10	0	0
	100	50	50
M. phlei	10	0	0
	100	100	100

[a] Groups of 10 mice each received intravenously the agent under test and D-galactosamine (20 mg) as a mixture in 0.2 ml of PBS. All controls, which received D-galactosamine or bacteria (up to 1 mg) or LPS only, survived. Lethality was scored up to 24 h after injection. Results comparable to the above were obtained in a second experiment with groups of six mice.

[b] NT, Not tested.

RESULTS

Lethal activity of LPS and of killed bacteria in D-galactosamine-treated mice. LPS of *S. abortus equi* and heat-killed gram-negative (*S. abortus equi* and *S. typhimurium*) and gram-positive (*Staphylococcus aureus*, *P. acnes*, and *M. phlei*) bacteria were tested for their lethal effects in D-galactosamine-treated mice. LPS-responder (C3H/HeN and C57BL/10 ScSN) and the respective congenic LPS-nonresponder (C3H/HeJ and C57BL/10 ScCR) mice were used. Groups of animals received different amounts of LPS (0.001 to 500.0 μg) or bacteria (0.1 to 1,000.0 μg) administered together with D-galactosamine (20 mg) intravenously. Table 1 summarizes the results obtained with C57BL/10 ScSN and C57BL/10 ScCR mice. As expected, purified LPS was highly toxic in the C57BL/10 ScSN mouse strain and nontoxic in the C57BL/10 ScCR strain. In the former, it caused 100% lethality at 0.01 μg and 20% lethality at 0.001 μg, while in the latter it caused no lethality in amounts up to 500 μg per animal. The parent *S. abortus equi* bacteria were toxic for C57BL/10 ScSN mice, 1 μg of bacteria causing 100% lethal-

ity and 0.1 μg having no lethal effect. A similar toxicity was obtained also by *S. typhimurium*-killed cells. This was not surprising since gram-negative bacteria contain LPS. However, in contrast to purified LPS, both gram-negative bacteria used were lethal also in C57BL/10 ScCR mice, 10 μg causing 20 to 30% lethality and 100 μg causing 100% lethality (Table 1). A 1-mg amount of the same bacteria administered in normal (not treated with D-galactosamine) mice of both strains caused no lethality.

Comparable results were obtained when the lethality tests with LPS and whole bacteria were carried out in C3H/HeN and C3H/HeJ mice.

The unexpected toxicity of whole bacterial cells for LPS-nonresponder mice prompted the extension of the study to gram-positive bacteria. D-Galactosamine-treated C57BL/10 ScSN and C57BL/10 ScCR mice, regardless of their sensitivity to LPS, exhibited a similar sensitivity to each of the gram-positive microorganisms used (Table 1). With 100 μg of *Staphylococcus aureus*, 100% lethality was obtained; with 10 μg, lethality was between 40 and 60%, while 1 μg was without lethal effect. *P. acnes* induced 50% lethality at 100 μg and 0% lethality at 10 μg. *M. phlei* induced 100% lethality at 100 μg and 0% at 10 μg. A 1-mg portion of the same bacteria caused no lethality in mice which had not received D-galactosamine.

Results very similar to those given above were obtained when the lethal effects of the gram-positive microorganisms were investigated in the congenic pair of mice, C3H/HeN and C3H/HeJ. Mice of both strains also exhibited equal sensitivity to each microorganism regardless of their sensitivity to LPS (data not shown).

Induction of TNF-α in C57BL/10 ScSN and C57BL/10 ScCR macrophages by LPS and by heat-killed bacteria in vitro. The ability of LPS and heat-killed bacteria (*S. abortus equi*, *S. typhimurium*, *Staphylococcus aureus*, *P. acnes*, and *M. phlei*) to induce TNF-α formation in macrophages of mouse strains C57BL/10 ScSN and C57BL/10 ScCR was investigated in vitro. Macrophages were obtained from bone marrow precursors (11). To stimulate TNF-α formation, 5×10^5 macrophages per ml were incubated with each agent for up to 24 h. Cell-free supernatants were tested for TNF-α activity in the L-929 cytotoxicity assay.

All agents tested induced the highest levels of TNF-α in culture supernatants within 2 to 4 h of incubation. Table 2 summarizes the data on TNF-α levels in 4-h supernatants. LPS induced a dose-dependent TNF-α only in C57BL/10 ScSN macrophages. Highest levels were obtained with the highest amount of LPS (0.2 μg) used for stimulation. No detectable TNF-α activity was present in supernatants of C57BL/10 ScCR macrophages (tested with up to 20 μg of LPS). *S. abortus equi* and *S. typhimurium* were, on a weight basis, as active as isolated LPS in their ability to induce formation of TNF in C57BL/10 ScSN macrophages. Bacteria at 0.002 μg sufficed to induce detectable amounts of TNF. In contrast to LPS, the gram-negative bacteria stimulated TNF-α also in C57BL/10 ScCR macrophages, although at a lesser efficacy; 0.2 μg was required to obtain detectable TNF levels. *Staphylococcus aureus* induced comparable levels of TNF-α in both C57BL/10 ScSN and C57BL/10 ScCR macrophages: 2 μg had to be added to obtain detectable TNF amounts. Very similar results were obtained in cultures of macrophages treated with *P. acnes* and *M. phlei*.

Like C57BL/10 ScCR macrophages, the C3H/HeJ macrophages were not stimulated by LPS to produce TNF. They could be stimulated, however, by the heat-killed gram-negative and gram-positive bacteria used in this study (data not shown).

TABLE 2. Induction of TNF-α by LPS and different heat-killed bacteria in LPS-responder (C57BL/10 ScSN) and LPS-nonresponder (C57BL/10 ScCR) macrophages[a]

Agent	Amt (μg/5×10^5 cells)	TNF-α (U/ml) produced by macrophages of:	
		C57BL/10	C57BL/10 ScCR
LPS			
S. abortus equi	20	NT	<4
	0.2	924	<4
	0.02	339	NT
	0.002	20	NT
Bacteria			
S. abortus equi	20	820	443
	2	1,000	151
	0.2	813	4
	0.02	227	<4
	0.002	9	NT
S. typhimurium	20	1,250	263
	2	1,140	131
	0.2	1,222	11
	0.002	20	NT
Staphylococcus aureus	20	439	1,694
	2	4	16
	0.2	<4	<4

[a] Macrophages at 5×10^5 per ml of culture medium were incubated with different amounts of the agents under test for 4 h at 37°C in a humidified atmosphere containing 8% CO_2. TNF activity in culture supernatants was estimated by the L-929 cell cytotoxicity assay with murine recombinant TNF-α as the standard. The results are representative of four different experiments carried out independently.

[b] NT, Not tested.

Neither LPS nor whole bacteria exhibited direct toxicity for the TNF-sensitive L-929 cell line. The TNF-α-positive macrophage supernatants did not show toxic effects on a TNF-α-resistant L-929 cell line.

Neutralization of cytotoxic activity in macrophage supernatants by anti-TNF-α serum. TNF-positive supernatants obtained after incubation of macrophages with the highest amount of the respective agent were incubated with the same volume of different dilutions of the anti-murine TNF-α serum or with normal rabbit serum (control) for 30 min at room temperature. Thereafter, each mixture was tested for TNF activity in the L-929 cell cytotoxicity assay. Under these conditions the cytotoxicity (200 U of TNF-α per ml) of the supernatants was completely inhibited by anti-TNF serum in a final dilution of 1:500, whereas the control rabbit serum was without effect. This shows that TNF-α is responsible for the cytotoxic activity of the supernatants.

Inhibition of the lethality induced by LPS and bacteria by anti-TNF-α serum. C57BL/10 ScSN mice received different amounts of rabbit anti-murine TNF-α serum. After 10 min the animals were challenged with lethal amounts of LPS (0.1 μg) or *S. abortus equi* (30 μg), *S. typhimurium* (30 μg), *Staphylococcus aureus* (100 μg), *P. acnes* (200 μg), and *M. phlei* (200 μg) administered intravenously together with D-galactosamine. Table 3 shows the results obtained with 10 and 100 μl of the antiserum. Whereas 10 μl of antiserum had no effect, 100 μl afforded complete protection in all cases.

TABLE 3. Inhibition of LPS- and bacterium-induced lethality by anti-TNF-α serum in C57BL/10 ScSN mice[a]

Challenge	Amt (μg)	Antiserum (μl)	Lethality (%)
LPS			
S. abortus equi	0.1	None	100
		10	100
		100	0
Bacteria			
S. abortus equi	30	None	100
		10	100
		100	1
S. typhimurium	30	None	100
		10	100
		100	0
Staphylococcus aureus	100	None	100
		10	100
		100	0
P. acnes	200	None	100
		10	100
		100	0
M. phlei	200	None	100
		10	100
		100	0

[a] Groups of five mice received, intravenously, the anti-TNF-α serum diluted in PBS (0.1 ml) and, 10 min thereafter, a mixture of the agent under test and D-galactosamine (20 mg) in 0.2 ml of PBS. Complete protection from the lethal activity of all bacteria was obtained in two further experiments with 100 μl of antiserum in groups of four mice.

Protection was also seen when the antiserum was administered up to 8 h before challenge, but it was absent when administered 1 h after challenge.

The anti-TNF-α serum also inhibited the lethal activity of *S. abortus equi* (300 μg), *S. typhimurium* (300 μg), *Staphylococcus aureus* (100 μg), *P. acnes* (200 μg), and *M. phlei* (200 μg) for D-galactosamine-treated C57BL/10 ScCR mice. Again, 100 μl of anti-TNF-α afforded complete protection, while 10 μl had no protective effect. A similar protective effect of anti-TNF serum towards the above bacteria was obtained in D-galactosamine-treated C3H/HeN and C3H/HeJ mice (data not shown).

Control mice receiving 100 μl of a normal rabbit serum before the lethal challenge were not protected.

DISCUSSION

Heat-killed gram-negative (*S. abortus equi* and *S. typhimurium*) and gram-positive (*Staphylococcus aureus*, *P. acnes*, and *M. phlei*) bacteria induced enhanced toxicity in D-galactosamine-treated mice. In addition, a number of other bacteria (*Bordetella pertussis*, *Brucella abortus*, *Escherichia coli*, *Streptococcus* sp. group A) were tested and found to exhibit lethal effects in this lethal toxicity model. As in earlier findings, in the present study, the lethal activity of purified LPS was expressed only in LPS-responder mouse strains (C57BL/10 ScSN and C3H/HeN). In contrast, all bacteria used, gram negative and positive, expressed lethal activity in LPS-responder and -nonresponder (C57BL/10 ScCR and C3H/HeJ) mice. The high toxicity of gram-negative bacteria for LPS-responder mice was to be expected since these bacteria contain LPS. LPS is generally believed to be the main biologically active component in gram-negative bacteria. Thus, in a recent study (19), it was shown that the property of gram-negative bacteria to induce macrophage Ia expression is due to their LPS content. The toxicity of gram-negative bacteria for LPS nonresponders is therefore surprising. It implies that gram-negative bacteria contain additional toxic components different from LPS, whose toxicity is expressed also in LPS-nonresponder mice. Since neither the identity nor the concentration of these components in the bacterial cell is known at present, a statement regarding their activity or a comparison with LPS cannot be made. The toxic component(s) appears to be heat stable since its activity survived the inactivation procedure (100°C/30 min) to which the bacterial cells had been subjected. The bacteria were significantly more toxic for LPS-responder than -nonresponder mice. This is explained by the predominant contribution of LPS to the lethal activity of the bacteria, which is expressed only in the responder animals.

The toxic principle in gram-positive bacteria is also not known. It seems to be heat stable, however, since it endured heat inactivation of the parent bacterial cells (100°C/30 min). Unlike gram-negative bacteria, gram-positive bacteria were equally toxic for all mouse strains regardless of their sensitivity to LPS. This is understandable because gram-positive bacteria do not synthesize LPS, which would express additional toxicity in the LPS-responder mice.

The use of D-galactosamine-treated mice for lethality tests represents a highly sensitive model, originally designed for measuring the toxicity of very low amounts of LPS (14). Meanwhile, it was shown that D-galactosamine does not increase sensitivity of mice towards the action of the LPS molecule itself, but rather towards the lethal activity of its endogenous mediator, most probably TNF-α (10, 18). This implies that, in principle, D-galactosamine-treated animals would be highly sensitive to the lethal action of any agent capable of inducing TNF-α formation. In the present study, the ability of the different agents to induce TNF-α formation was demonstrated in vitro in macrophages from LPS-responder and -nonresponder mice. In this way non-LPS-dependent responses to gram-negative bacteria may be differentiated, and the possibility that responses to gram-positive bacteria might be due to LPS contamination could be excluded. All gram-positive bacteria induced comparable levels of TNF-α in responder and nonresponder macrophages. Gram-negative bacteria induced more TNF-α in responder macrophages, evidently because of the additional activity of LPS they contain. Purified LPS, as expected, induced TNF-α formation only in LPS-responder macrophages. The results show that the absence of TNF formation in nonresponder macrophages treated with LPS does not reflect a general inability of the cells to synthesize this mediator. Responder and nonresponder macrophages are capable of producing TNF-α when stimulated with the appropriate agent. The ability of the agents (LPS and bacteria) to induce TNF-α in vitro correlates with their lethal activity in vivo.

The lethal activity of LPS for responder mice and that of gram-negative and -positive bacteria for responder or nonresponder mice could be completely inhibited by anti-murine TNF-α serum. In all cases 100 μl of the serum administered 8 h before, together with, or up to 30 min after a lethal challenge afforded complete protection. This result is evidence for the important role of TNF-α in mediating the lethality of LPS and of other toxic components present in gram-negative and gram-positive bacteria.

In addition to D-galactosamine, several other agents are

known to induce hypersensitivity to TNF-α (3, 21, 32). We have found that treatment with *P. acnes* also sensitizes mice to the lethal activity of gram-negative and -positive bacteria. Further, the lethal activity of all bacteria for *P. acnes*-infected mice could also be inhibited completely by anti-mouse TNF-α antiserum (results not shown). Thus, the property of the different bacteria to induce TNF-dependent lethal effects is not confined to D-galactosamine-sensitized mice.

The ability of some gram-positive bacteria to induce TNF-α formation in vitro has been reported (8, 9, 16, 17, 20, 35). Also, the presence of non-LPS components in gram-negative bacteria that are capable of stimulating cells from C3H/HeJ mice has been demonstrated. Thus, in an earlier study, Melchers et al. showed that the lipoprotein component and a number of other bacterial proteins induced stimulatory effects for B cells of LPS-nonresponder (C3H/HeJ) mice (22). These results were confirmed later by Morrison et al. (23), who used protein-containing LPS extracts. The property to exert stimulatory effects for B cells, however, is in no way a measure of the property of a preparation to induce shock. Consequently, the presence in gram-negative bacteria of a principle other than LPS that is capable of inducing TNF-α-dependent lethal shock has not been demonstrated.

The amount of TNF-α induced in D-galactosamine-treated mice by a lethal amount of LPS or killed bacteria is extremely low, being barely detectable by the usual assay methods. In nonsensitized animals, this amount would not be sufficient to induce lethality or to cause any ill effects whatsoever. Such small amounts of TNF-α, however, become extremely dangerous in hypersensitized animals, as shown in the present study. Sensitization to LPS or TNF-α may be established under different experimental conditions. A number of chemical agents and hepatotoxic substances as well as a number of growing tumors were shown to sensitize mice to the lethal action of TNF-α. Perhaps the most relevant sensitizing model is infection itself. Infection with different gram-negative bacteria was shown to sensitize mice to the lethal action of LPS and TNF-α (6, 12, 13, 21, 29). Also, a number of gram-positive bacteria (*P. acnes* and BCG) are known to exert similar sensitizing effects. Induction of hypersensitivity to TNF is therefore an important feature of bacterial infections because these not only possess the mechanism of TNF induction, but also can presensitize the host to its toxic activity. The present results suggest strongly that induction of TNF-α may be a common pathway in the development of lethal shock induced by gram-negative and gram-positive bacteria.

ACKNOWLEDGMENTS

We thank H. Stübig, C. Steidle, and M.-L. Gundelach for excellent technical assistance.

This study was supported in part by the Deutsche Forschungsgemeinschaft through SFB 154 and by the Bundesminister für Forschung und Technologie (grant 01 Ki 8809).

REFERENCES

1. **Agarwal, M. K., and G. Lazar.** 1984. Biological reactions to bacterial endotoxins in the non-responder C3H/HeJ mouse strain. Microbios **39**:193–207.
2. **Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goeddel, and R. N. Harkins.** 1985. Human tumor necrosis factor. Production, purification, and characterization. J. Biol. Chem. **260**:2345–2354.
3. **Bartholeyns, J., M. A. Freudenberg, and C. Galanos.** 1987. Growing tumors induce hypersensitivity to endotoxin and tumor necrosis factor. Infect. Immun. **55**:2230–2233.
4. **Beutler, B., and A. Cerami.** 1988. Cachectin/tumor necrosis factor: an endogenous mediator of shock and inflammation. Annu. Rev. Biochem. **57**:505–516.
5. **Beutler, B., I. W. Milsark, and A. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor (TNF) protects mice from the lethal effect of endotoxin. Science **229**:869–871.
6. **Chedid, L., and M. Parant.** 1971. Role of hypersensitivity and tolerance in reaction to endotoxins, p. 415–459. *In* S. Kadis, S. Weinbaum, and S. J. Ajl (ed.), Microbial toxins: a comprehensive treatise. Academic Press, Inc., New York.
7. **Coutinho, A., and T. Meo.** 1977. Genetic basis for unresponsiveness to lipopolysaccharide in C57BL/10 Cr mice. Immunogenetics **7**:17–24.
8. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1988. Nonpurulent response to toxic shock syndrome toxin 1-producing *Staphylococcus aureus*. J. Immunol. **140**:949–953.
9. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57**:291–294.
10. **Freudenberg, M. A., and C. Galanos.** 1988. Induction of tolerance to lipopolysaccharide (LPS)–D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. Infect. Immun. **56**:1352–1357.
11. **Freudenberg, M. A., D. Keppler, and C. Galanos.** 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. **51**:891–895.
12. **Galanos, C., M. A. Freudenberg, D. Krajewska, H. Takada, G. Georgiev, and J. Bartholeyns.** 1986. Endotoxin: structural aspects and immunobiology of host responses. EOS-J. Immunol. Immunopharmacol. **6**:78–86.
13. **Galanos, C., M. A. Freudenberg, D. Krajewska, H. Takada, G. Georgiev, and J. Bartholeyns.** 1988. Induction of lethality and tolerance by endotoxin are mediated by macrophages through tumor necrosis factor, p. 114–127. *In* B. Bonavida, G. E. Gifford, H. Kirchner, and L. J. Old (ed.), Tumor necrosis factor/cachectin and related cytokines. Karger AG, Basel.
14. **Galanos, C., M. A. Freudenberg, and W. Reutter.** 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76**:5939–5943.
15. **Galanos, C., O. Lüderitz, and O. Westphal.** 1979. Preparation and properties of a standardized lipopolysaccharide from *Salmonella abortus equi* (Novo-Pyrexal). Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **243**:226–244.
16. **Izumi, S., O. Hirai, K. Hayashi, Y. Konishi, M. Okuhara, M. Kohsaka, H. Aoki, and Y. Yamamura.** 1987. Induction of a tumor necrosis factor-like activity by *Nocardia rubra* cell wall skeleton. Cancer Res. **47**:1785–1792.
17. **Jupin, C., S. Anderson, C. Damais, J. E. Alouf, and M. Parant.** 1988. Toxic shock syndrome toxin 1 as an inducer of human tumor necrosis factors and γ interferon. J. Exp. Med. **167**:752–761.
18. **Lehmann, V., M. A. Freudenberg, and C. Galanos.** 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine treated mice. J. Exp. Med. **165**: 657–663.
19. **Marshal, N. E., and H. K. Ziegler.** 1989. Role of lipopolysaccharide in induction of Ia expression during infection with gram-negative bacteria. Infect. Immun. **57**:1556–1560.
20. **Mathison, J., and R. Ulevitch.** 1989. Workshop presentation, 7th International Congress of Immunology, Berlin.
21. **Matsuura, M., and C. Galanos.** 1990. Induction of hypersensitivity to endotoxin and tumor necrosis factor by sublethal infection with *Salmonella typhimurium*. Infect. Immun. **58**:935–937.
22. **Melchers, F., V. Braun, and C. Galanos.** 1975. The lipoprotein of the outer membrane of *Escherichia coli*, a B-lymphocyte mitogen. J. Exp. Med. **142**:473–482.
23. **Morrison, D. C., S. J. Betz, and D. M. Jacobs.** 1976. Isolation of

a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. J. Exp. Med. **144**:840–846.
24. **Morrison, D. C., and J. F. Ryan.** 1979. Bacterial endotoxins and host immune responses. Adv. Immunol. **28**:294–431.
25. **Old, L. J.** 1985. Tumor necrosis factor (TNF). Science **230**:630–632.
26. **Rosenstreich, D. L., and S. Vogel.** 1980. Central role of macrophages in the host response to endotoxin, p. 11–15. *In* D. Schlessinger (ed.), Microbiology—1980. American Society for Microbiology, Washington, D.C.
27. **Schramek, S., J. Kazar, L. Sekeyova, M. A. Freudenberg, and C. Galanos.** 1984. Induction of hypersensitivity to endotoxin in mice by *Coxiella burnetii*. Infect. Immun. **45**:713–717.
28. **Selye, H., B. Tuchweber, and L. Bertok.** 1966. Effect of lead acetate on the susceptibility of rats to bacterial endotoxins. J. Bacteriol. **91**:884–890.
29. **Seyberth, H. W., K. H. Schmidt-Gay, and E. Hackethal.** 1972. Toxicity, clearance and distribution of endotoxin in mice as influenced by actinomycin D, cycloheximide, α-amanitin and lead acetate. Toxicon **10**:491–500.
30. **Sultzer, B. M., and G. W. Goodman.** 1977. Characteristics of endotoxin-resistant low-responder mice, p. 304–309. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
31. **Tanamoto, K., U. Zähringer, G. R. McKenzie, C. Galanos, E.Th. Rietschel, O. Lüderitz, S. Kusumoto, and T. Shiba.** 1984. Biological activities of synthetic lipid A analogs: pyrogenicity, lethal toxicity, anticomplement activity, and induction of gelation of *Limulus* amoebocyte lysate. Infect. Immun. **44**:421–426.
32. **Wallach, D., Y. Nophar, D. Aderka, S. Israel, T. Hahn, H. Engelmann, and H. Holtmann.** 1988. Regulation of the response to tumor necrosis factor, p. 134–147. *In* B. Bonavida, G. E. Gifford, H. Kirchner, and L. J. Old (ed.), Tumor necrosis factor/cachectin and related cytokines. Karger AG, Basel.
33. **Wardle, E. N.** 1979. Bacteraemic and endotoxic shock. Br. J. Hosp. Med. **21**:223–231.
34. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. Teil B **7**:148–155.
35. **Yamamoto, A., M. Nagamuta, H. Usami, Y. Sugawara, N. Watanabe, Y. Niitsu, and I. Urushizaki.** 1985. A cytotoxic factor produced by OK-432. Immunol. Lett. **11**:83–88.