INTERFERON γ, A MEDIATOR OF LETHAL LIPOPOLYSACCHARIDE-INDUCED SHWARTZMAN-LIKE SHOCK REACTIONS IN MICE

By HUBERTINE HEREMANS, JO VAN DAMME, CHRIS DILLEN, ROGER DIJKMANS, AND ALFONS BILLIAU

From the Laboratory of Immunobiology, Rega Institute, University of Leuven, Medical School, 3000 Leuven, Belgium

LPS, a cell wall component of all Gram-negative bacteria, is well known to be responsible for a large part of the toxic manifestations associated with infections by such bacteria. When administered to experimental animals, LPS induces pathophysiologic changes that vary with the experimental conditions. A generalized and potentially lethal inflammatory reaction, known as the generalized Shwartzman reaction, can be elicited in several animal species, especially in rabbits (1), but also in mice (2). Typically, the reaction requires two consecutive, relatively low doses of LPS and is clinically manifested as a shock syndrome. The occurrence of the syndrome always requires careful dosage and timing (2). This reaction is of particular interest, since it shares pathogenetic elements with septic shock in humans, a condition that occurs with increasing frequency, and is a major cause of death among hospitalized patients in intensive care units.

Evidence from many studies indicates that the complex pathogenesis of septic shock and of the LPS-induced reactions is orchestrated by a complex network of endogenous mediators, whose release is triggered by the interaction of LPS with various cell types, especially phagocytes and endothelial cells. In recent years one particular group of mediators, the cytokines, has elicited considerable interest for their role in the LPS-induced inflammatory responses. Direct as well as indirect observations indicate that TNF-α and IL-1, two cytokines mainly produced by macrophages/monocytes, may be the principal mediators of the endotoxin-induced lethality. Thus, both these cytokines are released into the blood stream in humans (3, 4) or animals (3, 5) after intentional exposure to endotoxin, as well as during septic shock or meningococcal infection in patients (6-8). Furthermore, TNF induces profound shock, tissue injury, and death when infused into experimental animals (9-13). Moreover, a very strong synergism between TNF and bacteria or their products in causing a generalized Shwartzman-like reaction has been observed (14, 15). Finally, lethality

induced by LPS or Gram-negative bacteria can be inhibited by passive immunization with anti-TNF-α antibodies (9, 16, 17). Recently, it has also been demonstrated that IL-1 can markedly potentiate the lethal effect of recombinant TNF-α in mice (18, 19).

Nevertheless, TNF-α and IL-1 are certainly not the only major cytokines mediating septic shock. Additional factors seem to be IFN-γ and IL-6. IFN-γ is the principal macrophage activating factor released by activated T cells (20, 21). That it is involved in the pathogenesis of the inflammatory responses to LPS became evident from in vivo studies done in our laboratory, which demonstrate that local as well as generalized and lethal Shwartzman reactions elicited in mice can be completely prevented by pretreatment of the animals with mAb to IFN-γ (2, 22).

The studies reported in the present paper were done to gain more insight into the role of IFN-γ in the pathogenesis of the generalized Shwartzman reaction, and in particular to analyze its interaction with other cytokines, namely TNF-α, IL-6, and IFN-α and/or -β.

Materials and Methods

Mice. All experiments, unless otherwise stated, were done on female 7–8-wk-old NMRI mice bred under nonspecific pathogen–free (SPF)[1] conditions (Experimental Animal Breeding Facility, University of Leuven).

Reagents. Phenol-extracted LPS from *Serratia marcescens* was purchased from Sigma Chemical Co. (St. Louis, MO; Cat. no. L6136, lot 93F-4019). LPS derived from *Escherichia coli* (0111.B4) was purchased from Difco Laboratories (Detroit, MI). Stock solutions were prepared and diluted in PBS, pH 7.4. D-Galactosamine-hydrochloride was purchased from Janssen Biochimica (Beerse, Belgium).

Cytokine Preparations. Mouse IFN-α,β (MuIFN-α,β) was prepared on L929 cells infected with Newcastle disease virus (Komarov strain), concentrated, and partially purified by fractional precipitation with ammonium sulfate (23). The specific activity of the preparation used was $10^{6.8}$ U/mg protein. Recombinant murine IFN-α$_1$ (rMuIFN-α$_1$) was derived from CHO-psV10EF-3 cells (24), kindly provided by Dr. J. Trapman (Erasmus University, Rotterdam, The Netherlands). The interferon was purified by affinity chromatography on goat polyclonal antibodies against MuIFN-α,β giving preparations with a specific activity of $\sim 10^{7.3}$ (range: $10^{6.7}$–$10^{8.5}$) U/mg protein. Recombinant murine IFN-β (rMuIFN-β) was derived from CHO-βAA$_3$ cells, also kindly provided by Dr. J. Trapman. The interferon was purified by affinity chromatography on monoclonal anti-IFN-β (25), giving a preparation of $\sim 10^{8.3}$ (range: $10^{7.9}$–$10^{8.8}$) U/mg protein. rMuIFN-γ was derived from the supernatant fluid of Mick cells, a CHO cell line that carries and expresses an amplified murine IFN-γ cDNA (26). This interferon was purified by affinity chromatography on the F3 mAb to a specific activity of $\sim 10^{7.1}$ (range: $10^{6.5}$–$10^{8.0}$) U/mg protein. Dilutions of the cytokines were made in pyrogen-free saline.

Antibodies. mAbs against MuIFN-γ were obtained as ascites fluid from thymusless nude mice (*nu/nu*) inoculated with the (rat × mouse) hybridoma lines F1 and F3. The potential of both antibodies to bind IFN-γ and to neutralize its antiviral activity and macrophage-activating effect are described elsewhere (22, 27). The neutralizing potential of the F1 and F3 antibodies were $10^{-2.5}$ and $10^{-5.1}$ against 30 U/ml of MuIFN-γ, respectively (IgG content: F3, 2.5 mg/ml; F1, 5.3 mg/ml). Purified F3 mAbs (neutralizing titer of $10^{-5.75}$ against 30 U/ml) were obtained by affinity chromatography on an anti-rat κ chain mAb (MARK-1, reference 28). A mouse mAb against rat IFN-γ with binding and neutralizing activity for rat IFN-γ as well as MuIFN-γ (DB-1 cell line obtained by courtesy of Dr. Schellekens, TNO, Rijswijk, Netherlands) (29) was obtained as ascites fluid from BALB/c mice (neutralizing potential: $10^{-3.5}$ against 6 U/ml of rat IFN-γ; $10^{-2.5}$ against 30 U/ml of mouse IFN-γ). Control ascites fluid was obtained from nude mice injected with a rat × mouse hybridoma that

produces small amounts of irrelevant Ig (IgG content: 0.026 mg/ml; neutralizing titer of $<10^{-1.5}$ against 30 U/ml murine IFN-α, IFN-β, and IFN-γ). For administration in mice, all antibodies were diluted in pyrogen-free saline.

Induction of Generalized Shwartzman Reaction. The generalized Shwartzman reaction in mice was elicited by two consecutive injections of *S. marcescens* LPS: a preparatory one given in varying dose in the footpad followed after 24 h by a standard provoking dose of 100 μg intravenously. The occurrence of the generalized Shwartzman reaction was evaluated by morbidity scores and by mortality rates.

Assays for Biological Activity. Blood samples for determination on sera were taken from the orbital sinus and were allowed to clot at room temperature for about 1 h and at 4°C overnight. Serum samples were clarified and stored at -70°C until titration.

Interferon was titrated on primary mouse embryo fibroblasts (MEF) using a standard CPE inhibition assay with mengovirus as a challenge (23). Interferon activity was expressed in units per milliliter in terms of the NIH standard preparation G002-904-511. The presence of TNF did not interfere in the assay.

TNF levels in sera were determined using a cytotoxic assay on L929 cells as described (30). Briefly, serial dilutions (50 μl/well) of the serum samples were made in duplicate in 96-well microtiter plates (Nunc, Roskilde, Denmark) in EMEM supplemented with 2.2 g/liter sodium bicarbonate and 10% heat-inactivated FCS (Gibco Europe, Paisley, Scotland). To each well 100 μl of L929 cells (400,000/ml) in exponential growth were added in complete medium containing 2 μg/ml actinomycin D. After 18–24 h of incubation at 37°C the surviving cells were fixed and stained with 0.5% crystal violet (Merck) and the absorbance was measured in a Titertek at 590 nm. rMuTNF-α (Innogenetics, Gent, Belgium; sp act 4 $\times$ 10^7 U/mg protein) was included as a standard. The number of units/milliliter of activity was defined as the reciprocal of the dilution required to induce 50% decrease in absorbance relative to control cells exposed to actinomycin alone. The detection limit of the assay was 50–100 pg rTNF-α/ml serum. In some experiments a polyclonal rabbit antiserum directed against TNF-α (Innogenetics, Ghent, Belgium) was added to test the specificity of the toxicity.

IL-6 assays were done as described in Van Snick et al. (31) by incubating 2 $\times$ 10^3 IL-6-dependent hybridoma cells (mouse-mouse hybrid 7TD1) with serial dilutions of the test samples. After 4 d, viability was measured in a colorimetric assay with hexosaminidase. IL-6 concentrations ($\log_{10}$ units/ml) were calculated from titration endpoints. 1 U of IL-6 was defined as the amount required to obtain half-maximal growth of the cells. Pure natural IL-6 was included as an internal standard. The detection limit of the assay was 1–10 pg/ml.

Test for Endotoxin Contamination. Batches of IFN-γ and anti-IFN-γ antibodies were tested for endotoxin content by a chromogenic *Limulus amoebocyte* lysate assay (KabiVitrum, Stockholm, Sweden) and not used for in vivo experiments unless they contained <2 ng/ml.

Statistical Methods. Statistical methods were those used in a previous study (32).

Results

Induction of the Generalized Shwartzman Reaction in Mice: Suboptimal, Optimal, and Supraoptimal Induction Schedules. Groups of 4–6 mice were given two consecutive injections of *S. marcescens* LPS: a varying preparatory dose, followed after 24 h by a standard 100 μg provocative dose, the latter always given intravenously. The development of overt disease symptoms was then monitored over a period of 48–72 h. Table I shows that, depending on the route and size of the preparatory dose, the mice were sensitized towards the disease-inducing potential of the provocative dose. The symptoms consisted of pilo-erection (= disease score 0), lethargy, diarrhea, serous or hemorrhagic conjunctivitis (score 1), hemorrhagic lesions on ears and/or extremities (score 2), paralysis (score 3), and death (score 4).

Of various schedules tested, the optimal one was found to consist of a preparatory 5 μg dose in the footpad combined with a provocative 100 μg intravenous dose. At-

TABLE I

Incidence and Severity of Shock (Generalized Shwartzman Reaction) in Mice Given different LPS Treatment Schedules

Exp.	Preparatory LPS dose		Mean disease score (n)*		Mortality	
	Route	μg			Dead/total	Percent
1	Footpad	0	0.2	(4)	0/17	0
		0.5	1.2	(4)	0/19	0
		1.0	1.7	(1)	1/6	17
		5.0	3.5	(8)	32/38	84
		25	2.0	(4)	6/18	33
		50	1.0	(1)	1/4	25
2	Footpad	5	3.25	(1)	3/4	75
	i.v.	0.01	0.0	(1)	0/4	0
		0.1	0.0	(1)	0/4	0
		0.5	0.0	(1)	0/4	0
		1.0	1.4	(2)	2/8	25
		5.0	0.5	(1)	0/4	0
		10.0	0.0	(1)	0/4	0
	i.p.	0.01	1.5	(1)	0/4	0
		0.1	0.5	(1)	0/4	0
		0.5	0.25	(1)	0/4	0
		1.0	0.0	(1)	0/4	0
		5.0	0.5	(1)	0/4	0
		10.0	0.25	(1)	0/4	0
3	Footpad	5.0	3.13	(6)	28/38	(74)
	i.v.	10	0.3	(5)	1/32	(3)
	Footpad + i.v.	5.0 10.0	0.47	(6)	1/39	(3)

The generalized Shwartzman reaction was elicited in mice by two consecutive *S. marcescens* LPS injections, the preparatory one (varying dose and route as indicated) followed after 24 h by a standard 100 μg i.v. provoking dose.
* Figures represent means of average disease scores over n groups of mice (4–6 mice/group).

tempts to sensitize mice by intravenous or intraperitoneal rather than intra-footpad doses were unsuccessful. Preparatory doses smaller than the optimal 5 μg led to lesser disease symptoms and mortality; this will further be referred to as the suboptimal induction schedule. Preparatory doses higher than the optimal 5 μg also failed to sensitize the mice; this will further be referred to as the supra-optimal induction schedule. The ability of a 5 μg footpad dose to sensitize the mice was also abrogated by simultaneous intravenous injection of an additional 10 μg dose (Exp. 3 of Table I).

Cytokine Levels in the Serum of Mice with Generalized Shwartzman Reaction. In view of the apparent role of endogenous cytokines in the pathogenesis of the generalized Shwartzman reaction, it is of interest to know to what extent these cytokines are detectable in the general circulation. Therefore, blood samples taken from mice given the standard, suboptimal, or supraoptimal Shwartzman induction schedules were titrated for the presence of IFN, IL-6, and TNF.

Fig. 1 shows the course of serum IFN levels, as detected by antiviral effect on pri-
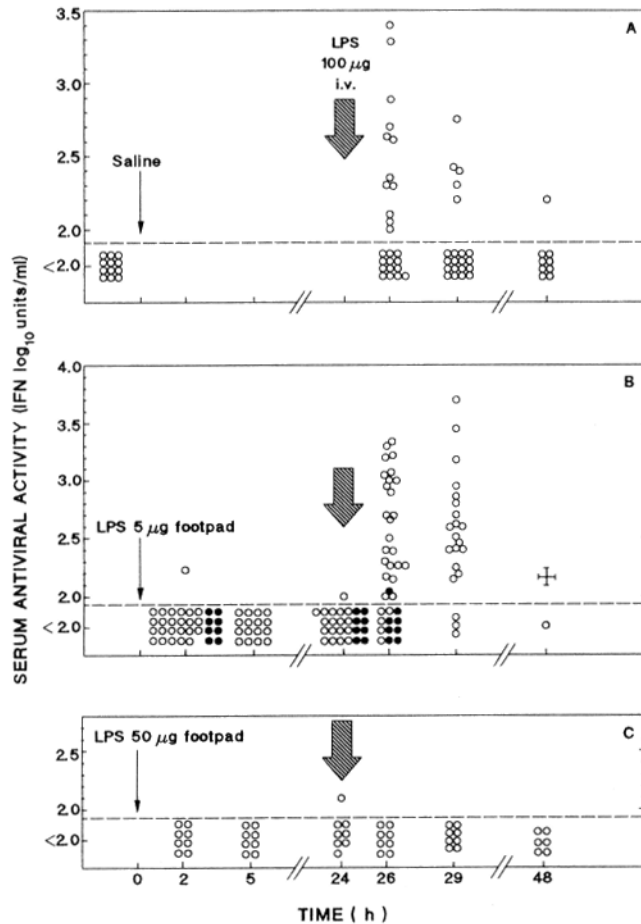
FIGURE 1. Course of serum IFN levels in mice given standard and supraoptimal Shwartzman induction schedules. All mice received a standard provoking dose of 100 μg *S. marcescens* LPS i.v. and varying preparatory doses in the footpad: (*A*) none; (*B*) optimal dose of 5 μg; (*C*) supraoptimal dose of 50 μg. (O) control mice; (●) anti-IFN-γ antibody treatment (F3 ascites, 0.1 ml i.p.) 24 h before the preparatory LPS dose. The sign ┼ in panel *B* indicates time point at which most mice have died.

mary mouse embryo fibroblasts. As can be seen in Fig. 1, the preparatory dose of LPS (5 or 50 μg in the footpad) did not itself result in detectable circulating IFN. The provoking dose (100 μg i.v.) induced high circulating IFN levels in about half of the mice that had not received any preparatory dose (panel *A*). In mice that had received an optimal preparatory dose (5 μg), the levels were similar, but persisted for a longer time (panel *B*, *open symbols*). Finally, in mice which had received a supraoptimal (50 μg) preparatory dose, the provoking dose failed to induce IFN (panel *C*). Similarly, in mice pretreated with anti-IFN-γ antibody, serum IFN remained undetectable (panel *B*, *filled symbols*).

The interferon-like antiviral activity measured was completely neutralizable by antiserum against IFN-α/β, but not by antibodies against IFN-γ or to TNF-α (neutralization data not shown). Although this indicates that the antiviral activity was predominantly due to IFN-α/β and not to TNF-α (alone or in synergy with IFN-α/β), the presence of a minor fraction of IFN-γ cannot be excluded.

Circulating TNF levels are shown in Fig. 2. TNF levels were detectable only at

a time point 2 h after injections of LPS. The 100 μg i.v. LPS injection induced TNF levels that were equally high, whether or not the mice had received the standard Shwartzman-preparatory LPS dose of 5 μg that is necessary for lethality to occur (panels A and B, *open symbols*). However, when the preparatory dose was supraoptimal (50 μg) so that the reaction was not lethal, TNF levels were low or undetectable (panel C). Also, in mice pretreated with anti-IFN-γ antibody, TNF remained undetectable (panel B, *filled symbols*). Cytotoxic activity in the serum was completely neutralizable by a polyclonal anti-TNF antibody (neutralization data not shown).

Circulating IL-6 levels in the generalized Shwartzman reaction are tabulated in Table II. As can be seen, little if any difference was found in the levels between mice given suboptimal, optimal, or supraoptimal induction schedules. Also, induction of IL-6 by a second dose of LPS was not subject to hyporeactivity as is the case for induction of IFN-γ or TNF-α (see Figs. 1 and 2). Furthermore, protection of the
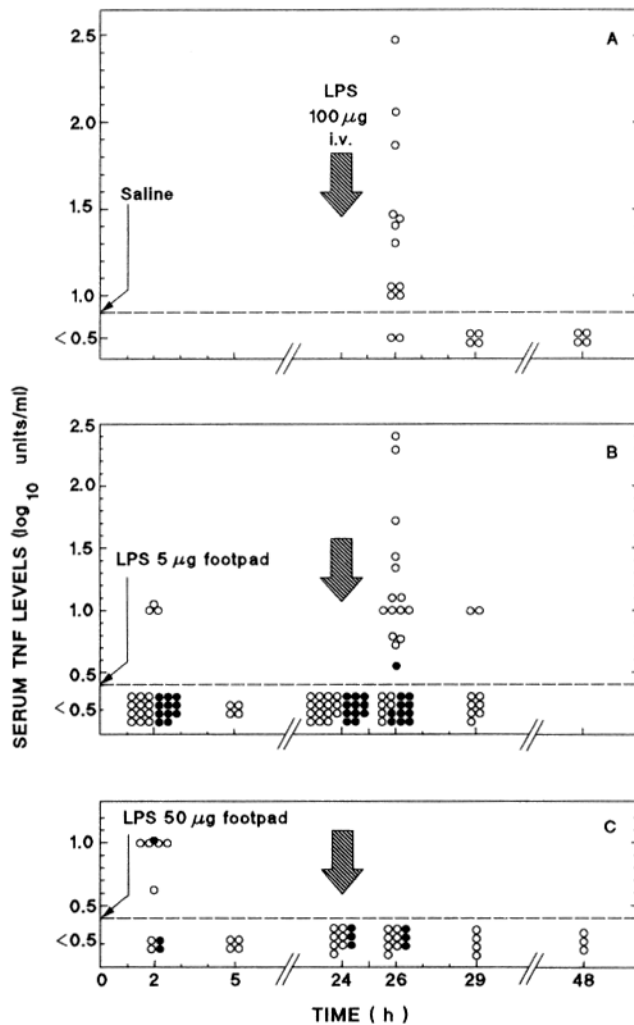

FIGURE 2. Course of TNF levels in sera of mice given standard and supraoptimal Shwartzman induction schedules. All mice received a standard provoking dose of 100 μg *S. marcescens* LPS i.v. and varying preparatory doses in the footpad: (A) none; (B) optimal dose of 5 μg; (C) supraoptimal dose of 50 μg. (O) control mice; (●) anti-IFN-γ antibody treatment (F3 ascites, 0.1 ml i.p.) 24 h before the preparatory LPS dose.

TABLE II

IL-6 Levels in Serum of Mice Given LPS Injections to Induce Generalized Shwartzman Reaction: Protective Regimens Are Not Associated with Lower IL-6 Levels

Treatment		IL-6 levels in serum at times (h): (log$_{10}$ units/ml, mean ± SE)		
Shwartzman induction-Prep. LPS Dose (μg)	Anti-IFN-γ antibody*	2	24	26
5 (n = 15)[‡]	−	2.88 (±0.07)	1.78 (±0.05)	4.21 (±0.08)
5 (n = 15)	+	2.63 (±0.09)	1.22 (±0.08)	3.92 (±0.10)
0.5 (n = 4)	−	2.75 (±0.06)	1.22 (±0.17)	4.42 (±0.10)
50 (n = 7)	−	3.88 (±0.06)	2.10 (±0.14)	3.98 (±0.07)
50 (n = 3)	+	3.80 (±0.11)	1.63 (±0.17)	3.67 (±0.03)
0 (n = 8)	−			4.10 (±0.15)

The generalized Shwartzman reaction was elicited in mice by two consecutive *S. marcescens* LPS injections, the preparatory one (varying dose as indicated) followed after 24 h by a standard 100 μg i.v. provoking dose.
* mAb F3, ascites fluid, 0.1 ml i.p. 24 h before preparatory LPS dose.
[‡] n, Number of independent serum samples tested.

mice by pretreatment with anti-IFN-γ antibody was not associated with lower levels of IL-6.

In conclusion, in comparisons of various lethal and nonlethal Shwartzman induction schedules, there seemed to be a rather good correlation between lethality and IFN or TNF levels and no correlation between lethality and IL-6 levels.

Protection of Mice Against the Generalized Shwartzman Reaction by Anti-IFN-γ Antibody. The involvement of IFN-γ in the pathogenesis of the generalized Shwartzman reaction was also studied by testing the effects of neutralizing anticytokine antibodies on the course of the disease. Fig. 3 shows the results of a series of experiments in which groups of mice were given an intraperitoneal injection of three different anti-IFN-γ mAb preparations, 24 h before the preparatory LPS dose of the standard Shwartzman induction schedule. All three monoclonals tested were found to be effective
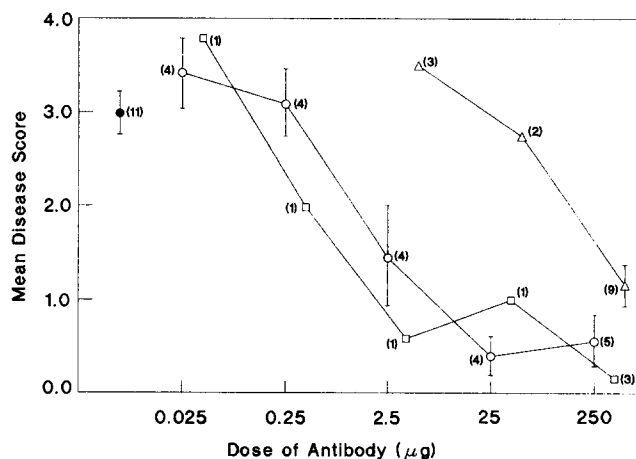

FIGURE 3. Prevention of generalized Shwartzman reaction in mice by treatment with anti-IFN-γ antibodies (dose-response curve). The reaction was elicited in mice by two consecutive *S. marcescens* LPS injections, a preparatory one (5 μg in the footpad) followed after 24 h by a provoking one of 100 μg i.v. (●) control; (△) F1 ascites; (○) F3 ascites; (□) DB1 ascites. Antibodies were given (0.1 ml i.p.) 24 h before the preparatory LPS dose. In parentheses number of experiments (4 mice per group) over which average disease scores are calculated.

in protecting the mice against the syndrome. mAb F1, which was the least effective is also known to be poorly neutralizing for IFN-γ in vitro (22).

Additional information on the protective effects of anti-IFN-γ antibodies is given in Table III. It can be seen that highly purified anti-IFN-γ antibody, F3, yielded protection similar to that provided by unpurified ascites fluid. Furthermore, protection afforded by a single injection of anti-IFN-γ antibody can be seen to last for more than 7 d.

Enhancing Effect of Exogenous IFN-γ on the Development of Generalized Shwartzman Reaction.   From the results of the experiments with anti-IFN-γ antibody it was hypothesized that the sensitizing effect of a preparatory LPS dose in the generalized Shwartzman reaction was mediated by IFN-γ induced by that dose. We therefore tested the ability of IFN-γ itself to prepare mice for a lethal Shwartzman reaction. To that end intraperitoneal injections of rMuIFN-γ were added to the suboptimal Shwartzman induction schedule. These injections were given 2 h after the preparatory LPS dose. Table IV shows the results of these experiments. It can be seen that IFN-γ treatment did indeed enhance the sensitivity of mice to the Shwartzman reaction, irrespective of whether it was given alone or in association with a suboptimal preparatory LPS dose. The effect was dose dependent, a minimal dose of $10^{4.7}$–$10^{5.0}$ U being required for the appearance of symptoms and $10^{5.7}$ U being required for lethality.

A time-response experiment (Table V) showed that IFN-γ was able to exert its sensitizing effect if it was given before the preparatory dose or between the preparatory and provocative LPS injections. It was found ineffective if given 2 h after the provocative dose.

Protective Effect of IFN-α/β towards Generalized Shwartzman Reactions in Mice.   LPS

TABLE III

Effect of Treatment with Different Anti-IFN-γ Antibody Preparations on the Severity and Mortality Rate of the Generalized Shwartzman Reaction Induced in Mice

Antibody*			Average disease		
Preparations	Time	n‡	score	Mortality	Percent
IFN-γ (F3 – asc)	Day – 1	8	0.65	2/37	5
IFN-γ (F3 – asc)	Day – 7	1	0	0/6	0
IFN-γ (F3 – pure)	Day – 1	1	0	0/4	0
IFN-γ (F1 – asc)	Day – 1	9	1.27	6/41	15
IFN-γ (DB1 – asc)	Day – 1	3	0.15	0/13	0
Saline		19	3.5	75/92	82

The generalized Shwartzman reaction was elicited in mice by two consecutive *S. marcescens* LPS injections, a preparatory one (5 μg in the footpad) followed after 24 h by a provoking one of 100 μg i.v.
* For origin and specificity, see Materials and Methods. Doses were as follows: F3 – asc: 5.6 $\log_{10}$ neutralizing units (NU) = 250 μg IgG; F3 – pure: 6.2 $\log_{10}$ NU = 200 μg IgG; F1 – asc: 3.3 $\log_{10}$ NU = 530 μg IgG; DB1 – asc: 3.3 $\log_{10}$ NU = 400 μg IgG.
‡ Number of groups (4–6 mice per group) over which average disease scores are calculated.

TABLE IV

Enhancing Effect of Systemically Administered IFN-γ on Development of LPS-induced Shock in the Generalized Shwartzman Reaction

Shwartzman induction schedule (preparatory dose of LPS, μg)	Dose* of IFN-γ (log₁₀ U/ml)	n‡	Average disease score (p value)§	Mortality number	
				Dead/total	Percent
5	5.7	2	3.62 (0.3)	7/8	88
	Nil		3.20	10/15	67
0.5	5.7	4	3.43 $(10^{-8.1})$	18/23	78
	Nil		0.74	0/23	0
0	5.7	2	2.55 $(10^{-4.8})$	4/9	44
	Nil		0.30	0/10	0
0.5	5.7	1	2.80 (0.009)	3/6	50
	5.0		1.67 (0.009)	0/6	0
	4.7		1.83 (0.009)	0/6	0
	4.0		1.00 (0.18)	0/6	0
	Nil		0.50	0/6	0

The generalized Shwartzman reaction was elicited in mice by two consecutive *S. marcescens* LPS injections, the preparatory one (varying dose as indicated) followed after 24 h by a standard 100 μg i.v. provoking dose.

* Intraperitoneally at time + 2 h relative to time of preparatory LPS injection.

‡ Number of experiments (each with groups of 4-6 mice) over which data are pooled.

§ Value in log rank of nul hypothesis for difference in score distributions between treated and "nil" groups.

induces various other cytokines, including IFN-α/β (see Fig. 1). Therefore, we wanted to test the effect of IFN-α/β on the occurrence of the Shwartzman reaction. Preliminary experiments having indicated that anti-IFN-α/β antibody failed to affect the reaction (data not shown), we tested the effect of IFN-α/β administered intraperitoneally at various time points in mice that were given the standard Shwartzman induction schedule. Table VI reveals a protective effect of a high dose ($10^{5.7}$ U/ml) of natural IFN-α/β, given 2 h after the preparatory dose of LPS. Table VI also shows that the observation was confirmed with recombinant MuIFN-α₁, but not with recombinant MuIFN-β.

Inability of Small Doses of LPS to Mimick the Protective Effects of Cytokines or Anticytokines. The experiments described in this paragraph were done to eliminate the possibility that the protective effects of anti-IFN-γ or the sensitizing effect of IFN-γ were due to contamination of the injected preparations with small amounts of LPS. Optimal as well as suboptimal induction schedules were used; two LPS preparations were taken as prototypes for possible contaminants (*E. coli* and *S. marcescens*); two doses were selected as being representative for possible contaminants (0.2 and 2 ng per injection); two injection times were selected, namely those at which preparations of anti-IFN-γ antibody and of IFN-γ had been found to provide in vivo effects. Table VII shows that under none of these circumstances was there any evidence that LPS injections mimick the effects of IFN-γ or of anti-IFN-γ antibody.

TABLE V

Enhancing Effect of Systemically Administered IFN-γ on Development of LPS-induced Shock in the Generalized Shwartzman Reaction: Time–Response Relationship

Time* (h) of IFN-γ injection ($10^{5.7}$ U i.p.)	Average disease score	Mortality number	
		Dead/total	Percent
− 2	2.67	6/12	50
+ 2	3.50	10/12	83
+ 22	2.83	3/6	50
+ 26	1.16	0/6	0
Saline	0.83	0/12	0

The generalized Shwartzman reaction was elicited in mice by two consecutive *S. marcescens* LPS injections, the preparatory one (suboptimal, 0.5 µg) followed after 24 h by a standard 100 µg i.v. provoking dose.

* Relative to time of preparative LPS dose.

Protection of Mice by Anti-IFN-γ Antibody against a Single Lethal LPS Dose; Failure to Protect Mice against Combined LPS + D-Galactosamine Treatment. Mice can be sensitized to the lethal effects of LPS not only by preparatory LPS injections, but also with different microorganisms and chemical agents (33) among which is D-galactosamine, a hepatotoxic agent, which specifically blocks hepatic RNA synthesis by depletion of free UTP (34). It was of interest to know whether anti-IFN-γ antibody would be able to protect mice sensitized to LPS by this drug.

To define suitable LPS doses for this type of experiment, we determined the LD_{50}

TABLE VI

Inhibitory Effect of Type I IFN (Natural α/β, Recombinant $\alpha1$, or Recombinant β) on Severity of the Shwartzman Reaction

IFN type	n*		Disease		Mortality	
			Score‡	p Value§	Score (%)	p Value‖
α/β	7	Untreated	3.58	—	39/45 (87)	—
		Treated¶	2.45	$10^{-9.1}$	20/42 (48)	0.009
α_1 (rec.)	3	Untreated	3.43	—	12/17 (71)	—
		Treated	1.58	$10^{-8.25}$	5/20 (25)	0.001
β (rec.)	3	Untreated	2.89	—	11/19 (58)	—
		Treated	2.91	NS	13/19 (68)	NS

The generalized Shwartzman reaction was elicited in mice by two consecutive *S. marcescens* LPS injections, a preparatory one (5 µg in the footpad) followed after 24 h by a provoking one of 100 µg i.v.

* Number of independent experiments, each with 5–9 mice per treatment group.

‡ Mean of average disease scores over n experiments.

§ Value in log rank of null hypothesis for difference in score distributions between treated and untreated groups.

‖ χ^2 test (χ^2 = sum, based on n degrees of freedom, of χ^2 values obtained for separate experiments).

¶ IFN treatment consisted of a single i.p. injection given 2 h after the preparatory LPS dose. Dose of natural IFN-α/β: $10^{5.7}$; recombinant IFN-$\alpha1$: $10^{5.2}$; recombinant β: $10^{5.3}$ U.

TABLE VII

Failure of LPS to Mimick Protective Effects of Cytokines or Anti-Cytokines against LPS-induced Shock in the Shwartzman Reaction

Exp.	Preparatory LPS dose (µg)	Additional LPS* injected Species	Time (h)	Dose (ng)	Average disease score	Mortality (dead/total)
1	5	S. marcescens	− 24	1	2.50	2/4
				0.2	3.75	3/4
				0	3.00	3/4
	0.5		+ 2	2	1.00	0/4
				0.2	0.75	0/4
				0	1.00	0/4
2	5	E. coli	− 24	1	3.20	3/4
				0.2	4.00	4/4
				0	3.25	3/4
	0.5		+ 2	2	1.25	0/4
				0.2	1.25	1/4
				0	0.75	0/4

The generalized Shwartzman reaction was elicited in mice by two consecutive S. marcescens LPS injections, the preparatory one (varying dose as indicated) followed after 24 h by a standard 100 µg i.v. provoking dose.
* Given i.p. at indicated times, relative to time of preparatory LPS dose.

of *E. coli* and of *S. marcescens* LPS in control and D-galactosamine–treated SPF NMRI mice. In accordance with reported findings (34, 35), simultaneous intravenous injection of mice with LPS and 20 mg D-galactosamine reduced the LD_{50} of LPS 75- to 330-fold (from 300 to 4 µg/mouse and from 233 to 0.7 µg/mouse for *S. marcescens* and *E. coli*, respectively). Next we tested the ability of anti-IFN-γ antibody to protect normal or D-galactosamine-sensitized mice. As can be seen in Table VIII, anti-IFN-γ antibody indeed provided near complete protection against a lethal dose (2

TABLE VIII

Effects of Pretreatment with Anti-IFN-γ Antibody on Morbidity and Mortality of Normal and D-Galactosamine-sensitized Mice, Given Lethal or Sublethal Intravenous Doses of LPS

Provocative intravenous LPS dose Species	µg	D-galactosamine*	Disease scores (and mortality) in mice pretreated[‡] with: Saline	Anti-IFN-γ[§]
S. marcescens	600	−	4.0 (6/6)	1.0 (0/6)
	100	+	2.2 (2/5)	3.0 (3/5)
	10	+	0.4 (0/5)	1.0 (1/5)
E. coli	500	−	4.0 (6/6)	1.0 (1/6)
	1	+	2.4 (3/5)	2.0 (2/5)
	0.1	+	1.6 (2/5)	1.6 (2/5)

* 20 mg given intravenously, simultaneously with the provocative LPS dose.
[‡] Pretreatments given 24 h before provocative LPS injection.
[§] mAb F3, ascites fluid, 0.1 ml i.p.

LD$_{50}$) of either toxin. However, it failed to affect morbidity or mortality in mice treated with the combination of D-galactosamine and LPS.

Discussion

In this study we have analyzed the involvement of several cytokines, especially of IFN-γ, in the generalized toxic effects of LPS in mice. Mice are known to be relatively resistant to induction of lethal shock by LPS: in our study the LD$_{50}$ of *E. coli* and *S. marcescens* endotoxins amounted to 233 and 300 μg/mouse, respectively. However, we have previously reported (2) and we confirm here that NMRI mice can be sensitized to the toxic effect of LPS by a small preparatory LPS dose (5 μg) given in the footpad 24 h before the intravenous provokative dose (100 μg). A 75–85% lethal shock-like syndrome is observed in such mice. Both the induction procedure and symptoms of this syndrome resemble those of the generalized Shwartzman reaction, as it has been described to occur in other animal species, particularly rabbits (1). We have therefore adopted the same term to denote the syndrome in mice.

Whereas a small local LPS dose (e.g., 5 μg) resulted in sensitization of the mice to a subsequent intravenous LPS challenge, a higher dose (e.g., 50 μg) did not have such an effect. One possible explanation for this biphasic response pattern is that, a high local doses, LPS may enter the circulation in sufficient quantities to cause a state of generalized hyporeactivity, as has been described in many reports (35). This interpretation gains support from the fact that the sensitizing effect of a local LPS dose could be undone by simultaneous intravenous injection of a relatively small LPS dose. Furthermore, in the mice receiving a supraoptimal preparatory dose, subsequent induction of circulating TNF and IFN-α/β was impaired (see below).

We also have used another known procedure for sensitization of mice to LPS, which consists in treatment with D-galactosamine (34): its simultaneous intravenous administration with *E. coli* or *S. marcescens* endotoxins reduced the LD$_{50}$ of these toxins 330- or 75-fold, respectively. The involvement of IFN-γ in these lethal reactions was studied by different approaches. Pretreatment of the mice with a single dose of a neutralizing mAb against IFN-γ prevented disease and death in mice given a single lethal LPS dose as well as in those given the generalized Shwartzman induction schedule. We interpret this to mean that IFN-γ is produced after LPS administration, and that this IFN-γ plays a crucial role in the pathogenesis of the lethal events following such injection. We also found that exogenous administration of IFN-γ by itself or in association with a small, local dose of LPS sensitizes the mice for the lethal effects of an intravenous challenge dose of LPS.

In contrast to mice given one lethal LPS dose, D-galactosamine–sensitized mice were not protected against LPS by pretreatment with anti-IFN-γ antibody, suggesting that in this case, the pathogenesis of lethal LPS-induced disease may be different and less (or perhaps not at all) dependent on the production of IFN-γ.

Circulating interferon-like antiviral activity was detectable in two-thirds of the mice given the generalized Shwartzman reaction schedule. However, this activity was identified as IFN-α/β rather than IFN-γ. Mice that failed to develop the reaction because they had not received the preparatory sensitizing LPS dose, had slightly lower IFN levels; mice that failed to develop the reaction because they had received a supraoptimal preparatory dose of LPS, or because they had received anti-IFN-γ antibody, produced no detectable circulating interferon.

To answer the question whether this IFN-α/β would have a positive or negative influence on the development of the lethal syndrome, we studied the influence of large doses of exogenous IFN-α/β on the development of the Shwartzman reaction. The natural IFN-α/β mixture as well as recombinant IFN-α_1, but not recombinant IFN-β, were found to inhibit the reaction and to reduce mortality. This suggests that systemic IFN-α, produced in association with the generalized Shwartzman reaction, acts as a mitigating factor on the syndrome.

Other investigators have provided evidence indicating that the cytokine which occupies a central place in the pathogenesis of LPS-induced shock reactions in rodents (9, 10), baboons (3, 16), and man (4, 6–8) is TNF. Another cytokine which has been incriminated in shock reactions due to Gram-negative sepsis is IL-6 (8, 36–38). Therefore we considered it essential to obtain information on the role of these cytokines in our model. Blood levels of TNF paralleled those of IFN-α/β: TNF was detected in mice programmed to die from a generalized Shwartzman reaction, but not in those protected by a supraoptimal preparatory dose of LPS or by anti-IFN-γ antibody. This close association between TNF levels and lethality, also observed in humans with Gram-negative septicemia (6–8), is consistent with an important role for TNF in the pathogenesis of the syndrome. From the evidence obtained with other model systems (5, 10–13, 39, 40) it appears most likely that TNF is a triggering factor responsible for much of the pathophysiological effects of LPS.

Blood levels of IL-6, in contrast to those of TNF and IFN-α/β did not correlate with severity of the shock syndrome: LPS induced high levels of IL-6 in all cases and kinetics were not different, whether the mice were programmed to develop a lethal syndrome or to be protected. These data are not confirming studies indicating an association between high serum levels of IL-6 and fatal outcome in patients with septic shock (8, 37). So far evidence is lacking for assigning a positive or negative role to IL-6 in LPS pathology. Perhaps its most important role consists in stimulating the immune response and in inducing hepatocytes to produce acute-phase reactants (41).

Our study indicates that endogenous IFN-γ plays a crucial role in the pathogenesis of lethal reactions to LPS. This raises several questions as to the substances, cells, and interactions involved. Classical target cells for LPS are monocytes, macrophages, and endothelial cells. According to recent evidence they respond not only to the LPS molecule itself but also to proteins associated with it (42). IFN-γ is well known to be a strong activator of phagocytic (43) and endothelial cells (44), both prolific producers of IL-1. Exogenous TNF can mimick the toxic effects of LPS (for reviews see references 45, 46). Furthermore, IFN-γ and TNF are known to synergize in various model systems (47, 48). Since treatment with anti-IFN-γ antibody not only prevented the lethal response, but also blocked the induction of circulating TNF associated with the lethal reactions, it seems logical to propose that IFN-γ constitutes a pathway by which LPS sensitizes animals to subsequent production and action of TNF. A quite similar situation has been observed in experimental cerebral malaria, in which anti-IFN-γ treatment also prevents mortality and the associated TNF production (49).

Whereas interaction with TNF seems the most plausible explanation for the sensitizing effect exerted by IFN-γ, there are other factors such as proteases, prostaglandins, leukotrienes, reactive oxygen and vasoactive peptides, all of which can

contribute to the toxic manifestations in animals exposed to LPS. The production and action of these factors may also be regulated by IFN-γ. It is possible therefore that further research will reveal additional in vivo pathways by which IFN-γ intervenes in LPS pathology. In particular, it will be intriguing to find out what role should be assigned to LPS-associated proteins (42) in the induction of IFN-γ as well as in the higher sensitivity to LPS of the IFN-γ–exposed host.

In clinical practice, LPS-induced shock is encountered as a complication in cases of Gram-negative sepsis. The frequency of this condition has been steadily increasing, due to the ever-increasing number of medical interventions that compromise host defenses, such as aggressive chemotherapy for cancer or organ transplantations (50). Furthermore, improved treatment for severely burned patients, has also led to an increasing number of such patients being at risk for Gram-negative sepsis. There is currently no effective treatment or prophylaxis for the occurrence of shock in these patients. The demonstration that cytokines such as TNF and IFN-γ are pivotal in the pathogenesis opens new perspectives for the development of novel strategies to prevent these complications.

Summary

The involvement of cytokines in the pathogenesis of a generalized, Shwartzman-like lethal inflammatory response to bacterial lipopolysaccharides (LPS) was studied by testing the ability of cytokines or neutralizing anticytokine antibodies to modify the course of the syndrome. The reaction was elicitable in non-SPF NMRI mice by two consecutive injections of S. marcescens LPS: a first injection in the footpad, followed after 24 h by an intravenous dose; the size and route of the preparatory LPS dose were found to be critical. Treatment with mAbs against IFN-γ was found to completely prevent the reaction. Treatment with IFN-γ on the other hand, rendered the mice more sensitive to elicitation of the reaction. In contrast, systemic administration of IFN-α/β exerted a desensitizing effect.

The role of endogenous cytokines in the pathogenesis of this generalized Shwartzman reaction was also documented by a study of the cytokine levels in the serum of the mice. In comparisons between mice given lethal and nonlethal induction schedules, a good correlation was found between mortality rates and height of IFN or TNF levels, but no correlation was seen with IL-6 levels. Also, in mice that were protected by anti-IFN-γ antibody, serum IFN and TNF were undetectable, whereas IL-6 levels were as high as in unprotected mice.

These data provide evidence that among the cytokines that govern the inflammatory response to LPS, endogenous IFN-γ occupies a key position. These findings therefore also open perspectives for clinical application of IFN-γ antagonists.

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