Interleukin 12, Interferon γ, and Tumor Necrosis Factor α Are the Key Cytokines of the Generalized Shwartzman Reaction

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Summary

The Shwartzman reaction is elicited by two injections of lipopolysaccharide (LPS) in mice. The priming LPS injection is given in the footpad, whereas the lethal LPS challenge is given intravenously 24 h later. The injection of interferon γ (IFN-γ) or interleukin 12 (IL-12) instead of the LPS priming injection induced the lethal reaction in mice further challenged with LPS. Antibodies against IFN-γ when given together with the priming agent, prevented the lethal reaction in mice primed with either LPS, IL-12, or IFN-γ. Antibodies against IL-12, when given together with the priming agent, prevented the lethal reaction in mice primed with either LPS or IL-12 but not with IFN-γ. These results strongly suggest that LPS induces the release of IL-12, that IL-12 induces the production of IFN-γ, and that IFN-γ is the cytokine that primes macrophages and other cell types. Upon LPS challenge, the lethal Shwartzman reaction is induced by a massive production of inflammatory cytokines that act on the target sites already sensitized by IFN-γ. If mixtures of TNF and IL-1 or mixtures of TNF and IFN-γ are used to challenge mice previously primed with IFN-γ or IL-12, mortality is induced. In the same conditions, the individual cytokines or a mixture of IL-1 and IFN-γ do not replace the LPS challenge. When the mice are primed with LPS, the combination of TNF, IL-1, and IFN-γ induced only a partial mortality incidence suggesting that the involvement of other LPS-induced factors.

B acterial LPS is a cell wall component of Gram-negative bacteria that is responsible for most of the toxic manifestations associated with bacterial infections. In mice, a lethal shock syndrome, known as the generalized Shwartzman reaction, can be elicited by two consecutive injections of LPS (for review see 1). A priming dose of LPS, injected intradermally in the footpad (f.p.)[1], is followed after 24 h by an intravenous challenge injection of LPS. After this challenge injection, which is not lethal per se, the mice die within the following 48 h from disseminated intravascular coagulation, vascular occlusion, hemorrhage, perivascular accumulation of leukocytes, and necrosis (2). This hypersensitivity reaction occurs only if the time interval between these injections is crucially fixed at between 18 and 24 h. There is no response when the intravenous challenge of LPS is given either earlier or later, or when the order of priming and challenge

injections is reversed. Finally, the reaction does not occur if both the LPS injections are intradermal or if the priming dose of LPS exceeds an optimum. The careful dosage and timing of the LPS injections and the need of specific routes of administration indicate that the Shwartzman reaction is elicited by induced endogenous factors acting in a precise time sequence. IFN-γ, TNF, and IL-1 are known to be involved in the pathogenesis of the generalized Shwartzman reaction. IFN-γ seems important during the priming phase (3), whereas TNF and IL-1 are thought to be the lethal effector molecules acting on target sites already sensitized by IFN-γ. The local injection of LPS presumably induces the release of IFN-γ, which activates macrophages to produce large amounts of TNF and IL-1 (4, 5). In addition, endothelial cells activated by IFN-γ, TNF, and IL-1, enhance their adherence properties to leukocytes and their capacity to release procoagulant and proinflammatory factors (5). It is not clear how LPS stimulates the release of IFN-γ, since the IFN-γ–producing cells, namely NK and T lymphocytes, are poorly responsive to LPS (6). Recently, a novel cytokine, IL-12, was characterized (7,

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[1] Abbreviations used in this paper: f.p., footpad; h, human; m, mouse; MIF, macrophage inhibiting factor; R, receptor.

8). This cytokine, which is released by LPS-stimulated macrophages, induces the production of IFN-γ in T and NK cells and initiates the differentiation of Th cells into the Th1 lineage (for review see 9). As Gram-negative bacteria can stimulate the production of IL-12 and induce a Th1 response (10), IL-12 could be the link between the injection of LPS and the endogenous production of IFN-γ that sensitizes mice to further contact with LPS. This study reports that IL-12 is the pivotal factor in the priming phase of the Shwartzman reaction. The activity of IL-12 is mediated through the induction of IFN-γ, and IFN-γ or IL-1 can synergize with TNF-α to elicit the lethal reaction.

Materials and Methods

Mice. NMRI female mice, 20–23 g body weight, were purchased from Biological Research Laboratory (Fullingsdorf, Switzerland) and maintained in our animal facilities under specified pathogen free conditions.

LPS. Serratia marcescens LPS (Sigma Chemical Co., St. Louis, MO) was dissolved in pyrogen-free saline to 10 mg/ml, sterilized by 0.22 μm filtration, aliquoted, and stored frozen at −20°C.

Cytokines. Recombinant human IFN-γ (hIFN-γ) and mouse IFN-γ (mIFN-γ) were expressed in *Escherichia coli* and purified according to Gray and Goeddel (11, 12) and Doebeli et al. (13). They display a specific antiviral activity of 8 $\times$ 10^7 U/mg and 1.2 $\times$ 10^7 U/mg of protein, respectively. The recombinant hIL-1 (3 $\times$ 10^8 U/mg of protein), and mIL-12 (6.3 $\times$ 10^6 U/mg of protein) were produced by Roche Research Center (Nutley, NJ) (14–16). Recombinant hTNF-α was expressed in *E. coli* and kindly provided by Dr. B. Wipf and Dr. U. Ettlin (F. Hoffman La Roche Ltd., Basel, Switzerland). hTNF-α binds to the mouse p55 TNF receptor (17). Recombinant hIL-6 (10^8 U/mg of protein) was from Dr. DiPadova (Sandoz, Basel, Switzerland).

Antibodies. Rat mAbs XMG1,2 and AN18 are IgG1 against mIFN-γ (18, 19). Rat mAb GR20 (20) is an IgG2a specific for mIFN-γ receptor (mIFN-γR). The mouse mAb γ73 and mAb γR99 are IgG1 directed against hIFN-γ and hIFN-γR, respectively (21, 22). The mAb 20C2 is a rat IgG1 neutralizing hIL-12 activity, whereas the rat mAbs 5C3 and C15.6.7 neutralize mIL-12 activity (23, 24). Goat IgG anti-mIL-12 and control goat IgG were produced at Roche Research Center (Nutley, NJ) (25). Irrelevant mouse and rat IgG were from Sigma Chemical Co.

Endotoxin Contamination. All reagents were checked for endotoxin contamination by the Limulus amoebocyte lysate assay given that 1 IU/ml is equal to 0.1 ng/ml of United States Pharmacopea standard *E. coli* endotoxin (26). All the antibodies, mIL-12, mIFN-γ, and hIL-1 contained <0.05 IU/mg protein, hTNF-α contained 12 IU/mg protein (0.06 IU for 5 μg TNF-α).

Induction of the Generalized Shwartzman Reaction in Mice. The generalized Shwartzman reaction was elicited by two consecutive injections of *S. marcescens* LPS (1). The priming injection was given in the f.p. and was followed 24 h later by a challenge LPS injection given intravenously. The optimal doses of LPS were determined for each batch of LPS and ranged between 1 and 5 μg for the f.p. injection and between 200 and 400 μg for the intravenous injection. When the reaction was elicited with cytokines, the priming injection was given intraperitoneally unless otherwise stated, the challenge injection was given intravenously. In all the experiments described, mAbs were injected intraperitoneally. Each experimental group includes from three to five mice. The occurrence of the generalized Shwartzman reaction was evaluated by mortality scores.

Statistical Analysis. Differences between the treatment groups was evaluated with student's *t* test.

Results

Induction of the Generalized Shwartzman Reaction in Mice. Groups of five NMRI mice received two consecutive injections of LPS. The priming injection was given in the f.p. and was followed 24 h later by the intravenous challenge injection. The reaction was lethal only if the mice received both the preparative and the challenge injections (87–100% mortality). A single f.p. or a single intravenous injection was ineffective.

Priming of the Mice/Replacement of LPS with IFN-γ. The role of IFN-γ in the pathogenesis of the Shwartzman reaction was investigated by replacing the priming LPS f.p. injection by IFN-γ (Table 1, Fig. 1). All the mice primed intraperitoneally with 10^6 U mIFN-γ (83 μg) died after the intravenous injection of LPS given 24 h later. The priming effect was dose dependent. In a representative experiment, 65 and 30% lethality were induced after priming with 10^5 U and 10^4 U mIFN-γ, respectively (Fig. 1). The priming effect of mIFN-γ was abrogated by boiling the protein (not shown) and was not due to an intrinsic toxicity of the cytokine (two injections of 10^6 U at 24-h intervals were ineffective, see Table 4). Control animals, primed with hIFN-γ, all survived the LPS challenge (data not shown). These results show that IFN-γ can replace LPS in priming the mice to the Shwartzman reaction.

Priming of the Mice/Replacement of LPS with IL-12. Since IL-12 induces the secretion of IFN-γ, we assessed the hypothesis that IL-12 can mimic the LPS priming effect. Table 1 displays the results of the experiments where either the LPS priming f.p. injection or the LPS intravenous challenge injection were replaced by mIL-12. When mice were primed with 10 ng mIL-12 and challenged 24 h later with LPS, 85% died (Fig. 1). This priming effect of mIL-12 was dose dependent (17 and 0% mortality for 1 and 0.1 ng mIL-12, respectively) and was abrogated by boiling the protein (data not shown). The lethality was not due to an intrinsic toxicity of the cytokine. A single injection of 25 μg mIL-12 or two consecutive injections of mIL-12 with a 24-h time interval were ineffective (see Table 4). These results show that mIL-12 can replace the LPS priming in the Shwartzman reaction and that mIL-12 is 1,000 times more efficient than mIFN-γ in priming mice (Table 1, Fig. 1).

Antibodies against IFN-γ Inhibit the Shwartzman Reaction when the Mice Are Primed with LPS, IFN-γ, or IL-12. The roles of IFN-γ and IL-12 in the pathogenesis of the Shwartzman's reaction were further investigated using a mAb (XMG1,2) that neutralizes mIFN-γ activity (Table 2). A dose of 10 μg mAb XMG1,2 rescued mice from the shock reaction when given intraperitoneally simultaneously with the priming LPS injection (mortality reduced to 10%). Irrelevant rat IgG, mAb γ73 anti–human IFN-γ or mAb γR99 anti–human IFN-γR were not effective (Table 2). These results were further confirmed using AN18, another mAb anti-IFN-γ (data not

Table 1. *Both mIFN-γ or mIL-12 Can Induce the Shwartzman Reaction*

Priming f.p. 0 h		Priming intraperitoneally 0 h		LPS challenge intravenously 24 h	No. of experiments	No. of mice dead/tested	Mortality
							%
−		−		+	1	0/5	0
LPS	5 µg	−		+	2	6/10	60
−		LPS	5 µg	+	1	0/5	0
mIFN-γ	6 × 10³ U			+	1	0/5	0
mIFN-γ	6 × 10⁴ U			+	2	0/10	0
mIFN-γ	1.2 × 10⁵ U			+	1	3/5	60
		mIFN-γ	6 × 10³ U	+	1	0/5	0
		mIFN-γ	6 × 10⁴ U	+	2	6/10	60
		mIFN-γ	1.2 × 10⁵ U	+	1	2/5	40
mIL-12	1 ng			+	1	0/5	0
mIL-12	10 ng			+	2	10/10	100
		mIL-12	1 ng	+	1	0/5	0
		mIL-12	10 ng	+	2	7/10	70

For priming, mIL-12 and mIFN-γ can be injected either intraperitoneally or in the f.p.

shown) or GR20 a mAb raised against the mIFN-γ (Table 2). The antibodies to IFN-γ inhibited more efficiently the lethal reaction than antibodies to IFN-γR. The dosages giving 50% protection were 1 and 30 µg for XMG-1,2 and AN18,

Figure 1. Comparison of the efficiency of mIL-12 (□) and mIFN-γ (△) to prime intraperitoneally the mice for the induction of the lethal reaction after the LPS challenge. Each treatment group consists of five mice and four individual experiments were performed. The data of a representative experiment are shown and the results are expressed in percentage of mortality.

respectively, whereas 200 µg of GR20 were needed to reach the same protection level. This difference can be explained by the need to block all the mIFN-γ receptors which are ubiquitously expressed.

When the mice were primed intraperitoneally with 10⁵ U mIFN-γ, 87% of the mice died after the LPS challenge (Fig. 2). This lethal reaction was partially inhibited (mortality reduced to 40%) by the injection of 100 µg anti-IFN-γ mAb XMG-1,2 (Fig. 2 A). About 5 µg of the same mAb gave a complete inhibition of the lethal reaction developed by mice primed with 10 ng mIL-12 and challenged with LPS. The amount of XMG-1,2 that protects the mice primed with mIFN-γ is 10–20-fold higher than the amount needed to reach the same protection level in mice primed with LPS or mIL-12 (Fig. 2 A). It can be explained by the high dose of mIFN-γ (10⁵ U = 8.3 µg) that has to be used to prime the mice. These results suggest that both the priming effects of LPS and mIL-12 are mediated by mIFN-γ.

Antibodies against IL-12 Inhibit the Schwartzman Reaction when the Mice Are Primed with LPS, IL-12, but not IFN-γ. When given simultaneously with the LPS priming injection, goat anti-mIL-12 inhibited the lethal reaction (Fig. 2 B). A complete protection from lethality was obtained after intraperitoneal injection of 200 µg goat anti-mIL-12. This result was confirmed that using mAb 5C3 recognizes the 40-kD chain of the mIL-12 (mortality reduced to 20%), whereas mAb 20C2 raised against hIL-12 was almost ineffective (Table 3). To confirm that the priming effect of mIL-12 is IFN-γ dependent, we assessed the capacity of anti-IL-12 antibodies to inhibit the lethal reaction when the mice are primed with
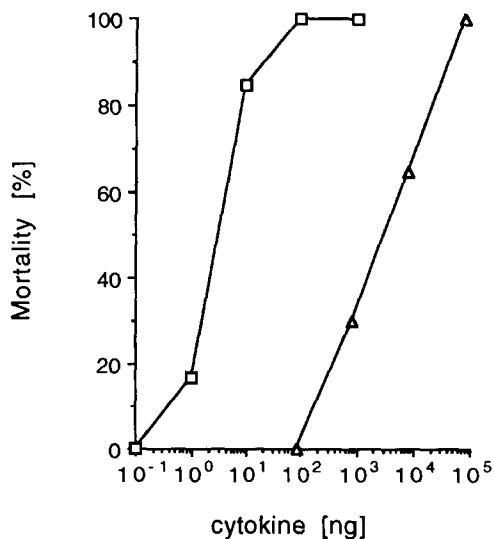
Table 2. *IFN-γ is involved in the Shwartzman reaction*

LPS priming f.p. 0 h	Ab treatment intraperitoneally 0 h		LPS challenge intravenous 24 h	Ab treatment intraperitoneally 24 h		No. of experiments	No. of mice dead/tested	Mortality
								%
−	−		−	−		2	0/10	0
+	−		+	−		20	89/98	91
+	γ73	100 μg	+	−		1	5/5	100
+	γR99	200 μg	+	−		1	5/5	100
+	Rat Ig	200 μg	+	−		2	9/10	90
+	XMG1,2	10 μg	+	−		19	10/95	10
+	GR-20	200 μg	+	−		7	8/35	23
+	−		+	γR99	200 μg	1	4/5	80
+	−		+	XMG1,2	10 μg	8	13/44	29
+	−		+	GR20	200 μg	3	4/15	27

The anti-IFN-γ or anti-IFN-γR antibodies given intraperitoneally together with the LPS f.p. injection (0 h) or with the LPS intravenous injection (24 h) prevent the lethal reaction. XMG1,2 is a rat anti-mIFN-γ mAb and GR-20 is a rat anti-mIFN-γR mAb. γ73 is a mouse anti-hIFN-γ mAb and γR99 is a mouse anti-hIFN-γR mAb. Rat Ig is an irrelevant rat IgG.

LPS, mIL-12, or mIFN-γ (Fig. 2 B). The goat anti-mIL-12 antibody efficiently inhibited the Shwartzman reaction when mice were primed with mIL-12 (IC50 ranging between 10 and 20 μg/mouse). When the mice were primed with 10^5 U mIFN-γ, the simultaneous injection of 200 μg of antibodies to mIL-12 only marginally reduced the mortality induced by the LPS challenge (Fig. 2 B). Altogether these results confirm that the priming injection of LPS induces the release of mIL-12 which in turns stimulates the production of mIFN-γ.
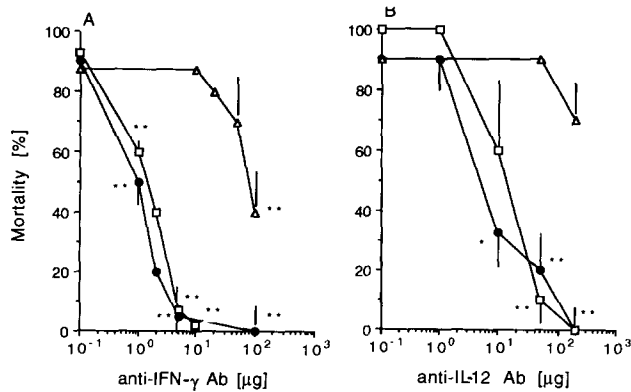
IFN-γ But Not IL-12 Has a Systemic Role in the Shwartzman Reaction. The priming injection of LPS was efficient if the injection was performed in the f.p. The same dose of LPS injected intraperitoneally was ineffective. In contrast, the priming of mice with mIFN-γ or with mIL-12 was similarly efficient when given either in the f.p. or intraperitoneally (Table 1). For both cytokines, no significant difference in the priming dose was found when the two routes of administration were compared. In these experiments, a priming dose of 10 ng mIL-12 infected in the f.p. or intraperitoneally 24 h before the LPS challenge, induced 100 and 70% mortality,
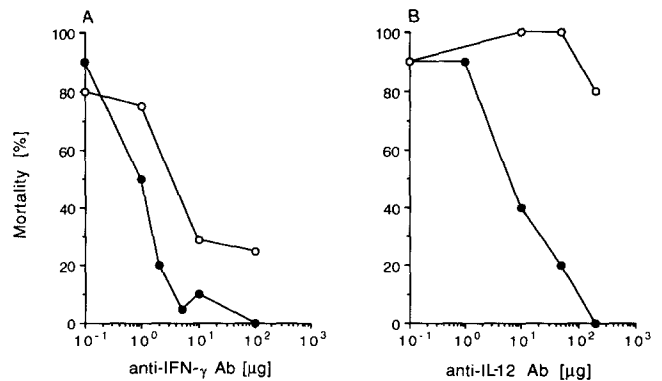

Figure 2. Test of the inhibitory efficiency of mAb XMG1,2 anti-IFN-γ (A) or goat Ab anti-IL-12 (B) in the lethal Shwartzman reaction when given intraperitoneally at time 0 h together with the priming agent. Mice f.p. were primed with LPS (●), with 10 ng mIL-12 intraperitoneally (□), or with 0.83 μg (10^5 U) mIFN-γ intraperitoneally (△) and challenged intravenously with LPS 24 h later. The results are expressed as the mean percentage of mortality obtained in three to ten individual experiments with five mice per treatment group. Standard errors (*vertical bars*) and p values (*p < 0.01, **p < 0.001) are indicated.

Figure 3. Comparison of the inhibitory efficiency of mAb XMG1,2 anti-IFN-γ (A) or goat Ab anti-IL-12 (B) in the lethal Shwartzman reaction when given intraperitoneally either at time 0 h together with the LPS priming (●) or at time 24 h together with the LPS intravenous challenge (O). Data are pooled from three to eight independent experiments with five mice per treatment group. The results are expressed in percentage of mortality.

Table 3. *mIL-12 Is Involved in the Shwartzman Reaction*

LPS priming f.p. 0 h	Ab treatment intraperitoneal 0 h		LPS challenge intravenous 24 h	Ab treatment intraperitoneal 24 h		No. of experiments	No. of mice dead/tested	Mortality
								%
−	−		−	−		2	0/10	0
+	−		+	−		4	18/20	90
+	mIgG	200 μg	+	−		1	5/5	100
+	rat IgG	200 μg	+	−		1	4/5	80
+	goat IgG	200 μg	+	−		1	5/5	0
+	5C3	200 μg	+	−		2	2/10	20
+	C15.6.7	100 μg	+	−		2	0/10	0
+	20C2	200 μg	+	−		1	4/5	80
+	GαmIL-12	200 μg	+	−		2	0/10	0
+	−		+	goat IgG	200 μg	1	4/5	80
+	−		+	GαmIL-12	200 μg	2	8/10	80

The antibodies anti-mIL-12 given intraperitoneally together with the LPS f.p. injection (0 h) prevent the lethal reaction. 5C3 and C15.6.7 are rat anti-mIL-12 mAbs, 20C2 is a mouse anti–human IL-12 mAb. GαmIL-12 is a goat anti-mIL-12 polyclonal antibody. mIgG, rat Ig and goat IgG are irrelevant mouse, rat IgG, or goat IgG.

respectively. When 1.2 × 10^{5} U (10 μg) mIFN-γ were used to prime mice via the f.p. or intraperitoneally, the mortality induced by the LPS challenge was 60 and 40%, respectively. Since mIL-12 or mIFN-γ efficiently primed the mice when given intraperitoneally, these cytokines may play a systemic role in the Shwartzman reaction. We assessed this hypothesis by injecting goat anti-mIL-12 antibodies or anti-IFN-γ mAb XMG 1,2 together with the intravenous LPS challenge. When the XMG1,2 antibody was given simultaneously with the LPS challenge (Fig. 3 *A*, Table 2), a significant protection from lethal shock was still observed (100 μg/mouse reduced the mortality to 23%). In the same conditions, 200 μg of

goat anti-mIL-12 antibodies were ineffective (Fig. 3 *B*, Table 3). However, neither mIFN-γ nor mIL-12 could replace the challenge LPS injection of the Shwartzman reaction: the mice that were primed with LPS and challenged intravenously 24 h later with 10[6] U mIFN-γ or 0.1 μg mIL-12, survived (Table 4). Since the mIL-12 priming effects are mediated by IFN-γ, these experiments confirm that mIFN-γ plays a role in the lethal reaction.

Toxicity of TNF-α, IL-1, and IFN-γ Injected Intravenously. To evaluate the role of IFN-γ, IL-1, and TNF-α in the challenge phase of the Shwartzman reaction, the toxicity of the cytokines was assessed after a single intravenous injection.

Table 4. *Challenge with Single Cytokines in the Shwartzman Reaction*

Priming 0 h		Challenge with cytokine intravenous 24 h		No. of experiments	No. of mice dead/tested	Mortality
						%
	LPS	IL-1	10[6] U	1	0/5	0
	LPS	IL-6	10[6] U	1	0/5	0
	LPS	IL-12	0.1 μg	2	0/8	0
	LPS	IFN-γ	10[6] U	2	0/8	0
	LPS	TNF	5 μg	2	0/6	0
IL-12	0.01 μg	IL-12	0.1 μg	1	0/5	0
IFN-γ	10[6] U	IFN-γ	10[6] U	1	0/5	0

Mice primed with LPS and challenged intravenously with single cytokines do not develop the lethal reaction.

Doses of up to 10 μg TNF-α, 10^5 U IL-1, or 10^5 U mIFN-γ, when injected alone, did not induce mortality (Tables 4 and 5). The coinjection of 10^5 U IL-1 and 10^5 U IFN-γ was neither toxic. On the contrary, TNF-α coinjected with either IL-1 or IFN-γ could induce mortality (Table 5). Combinations of 5 μg TNF-α and 10^4 U IFN-γ or IL-1 and combinations of 2 μg TNF-α and 10^5 U IFN-γ or IL-1 were not toxic in normal mice. When the three cytokines were coinjected (Table 6), 2 μg TNF-α together with 10^4 IL-1 and 10^5 U IFN-γ were toxic, whereas 2 μg TNF-α together with 10^5 IL-1 and 10^4 U IFN-γ did not induce lethality. The dosages of cytokines applied to challenge mice in the Shwartzman reaction were chosen according to their lack of toxicity as defined in the Tables 5 and 6.

Challenge of Mice with Cytokines after Priming with LPS. Mice primed with LPS and challenged intravenously with either TNF-α (5 μg), IL-1 (10^6 U), IL-6 (10^6 U), or mIFN-γ (10^6 U) did not die (Table 4). Neither the coinjection of two cytokines, TNF-α and IL-1, TNF-α and mIFN-γ, or IL-1 and mIFN-γ, could replace the LPS challenge in LPS-primed mice (Fig. 4 *A*). The challenges with 2 μg TNF-α, 10^4 U IL-1, and 10^4 U mIFN-γ or with 1 μg TNF-α, 10^4 U IL-1, and 10^5 U mIFN-γ induced 33% mortality in mice previously primed with LPS. The mortality incidence reached 50% in mice primed with LPS and challenged with 2 μg TNF-α, 10^5 U IL-1, and 10^4 U mIFN-γ.

These experiments show that cytokines can induce the lethal Shwartzman reaction in the LPS primed mice; that at least TNF-α, IL-1, and mIFN-γ can elicit the reaction; and that other factors may be necessary to reach 100% mortality incidence.

Challenge of Mice with Cytokines after Priming with IFN-γ or IL-12. When mice were primed with 10^5 U mIFN-γ or with 10 ng mIL-12 and challenged with single cytokines such as TNF-α, IL-1, IL-6, or mIFN-γ, no mortality was observed (not shown). Fig. 4 shows that the coinjection of two

cytokines, either TNF-α and mIFN-γ or TNF-α and IL-1, but not IL-1 and mIFN-γ, can elicit the lethal reaction in mice primed with mIFN-γ or mIL-12. The highest mortality incidence was obtained when mice primed with cytokines were challenged with TNF-α and IFN-γ. The intravenous injection of 5 μg TNF-α and 10^4 U mIFN-γ induced 83 and 100% mortality in mice primed with mIL-12 and mIFN-γ, respectively. In comparison, the injection of 5 μg TNF-α and 10^4 U IL-1 was less efficient and induced 33 and 83% mortality in mice primed with mIL-12 or mIFN-γ, respectively. The injection of 2 μg TNF-α and 10^5 U mIFN-γ was lethal only in mice primed with mIFN-γ (67% mortality), whereas the injection of 2 μg TNF-α and 10^5 U IL-1 was not effective whichever the priming cytokine.

When the three cytokines were injected to challenge the mice primed with mIFN-γ or mIL-12, the minimal doses giving high mortality incidence were either 2 μg TNF-α, 10^4 U IL-1, and 10^4 U mIFN-γ or 1 μg TNF-α, 10^4 U IL-1, and 10^5 U mIFN-γ (100% mortality in mice primed with mIFN-γ and 83–100% in mice primed with mIL-12). The decrease from 10^4 to 10^3 U of the IL-1 present in the cytokine mixture injected, reduced drastically the lethality of the reaction (0 and 22% mortality in mice primed with IL-12 and IFN-γ, respectively). On the contrary, the mortality rates did not change significantly when the mIFN-γ present in the cytokine combination was reduced from 10^4 to 10^3 U (76 and 83% mortality in mice primed with IL-12 and IFN-γ, respectively).

These experiments show that TNF-α when coinjected with IL-1 or mIFN-γ can induce the lethal Shwartzman reaction in the mIL-12 or mIFN-γ primed mice and that TNF-α is essential for the lethality of the reaction, whereas IL-1 or mIFN-γ potentiate the TNF-α effects. Finally, these experiments show that both mIL-12 and mIFN-γ are more efficient priming agents than LPS in the lethal Shwartzman reaction.

Table 5. *Toxicity of TNF-α when Coinjected Intravenously with IL-1 or mIFN-γ*

		μg of TNFα			
		0	2	5	10
	0	0	0	0	0
Units of IL-1	10^4	0	nt*	0	0
	10^5	0	0	67	100
Units of IFN-γ	10^4	0	nt	0	67
	10^5	0	0	50	nt

The results from two individual experiments are expressed in percent of mortality. In each experiment, each treatment group includes three to five mice.
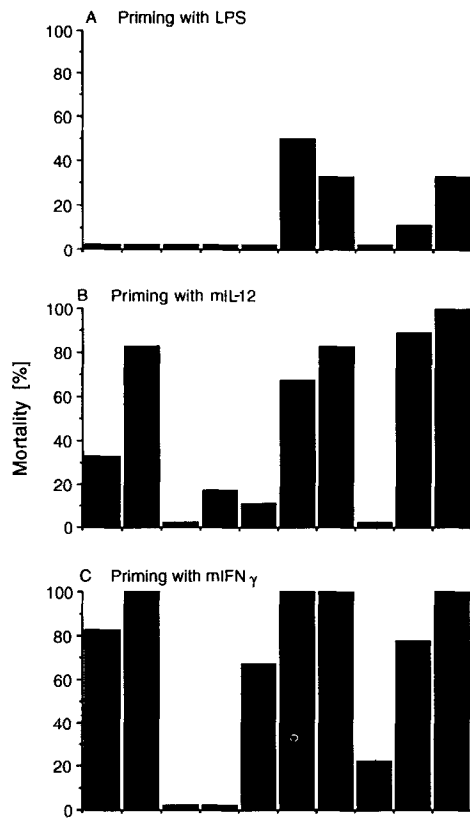* Not tested.

Table 6. *Toxicity of TNF-α when Coinjected Intravenously with IL-1 and IFN-γ*

		μg of TNF-α					
		0	1	2	2	2	5
Units of IL-1		10^6	10^4	10^3	10^4	10^5	10^4
Units of IFN-γ	0					0	0
	10^3				0	0	nt*
	10^4			0	0	0	50
	10^5	0	0	0	33	100	100

The results from two to six individual experiments are expressed in percent of mortality.
In each experiment, each treatment group includes three to five mice.
* Not tested.

TNFα [µg]	5	5	–	2	2	2	2	2	2	1
IL-1 [x 10⁴U]	1	–	1	10	–	10	1	0.1	1	1
IFNγ [x 10⁴U]	–	1	1	–	10	1	1	1	0.1	10

Figure 4. Test of the challenge efficiency of TNF-α, IL-1, and mIFN-γ in primed mice. Mice were primed either with 5 µg LPS in the f.p. (*A*), with 10 ng mIL-12 intraperitoneally (*B*) or with 10⁵ U mIFN-γ intraperitoneally (*C*). The doses of cytokines given to challenge primed mice were not toxic as defined in Tables 4 and 5. Data are pooled from three to five independent experiments with three mice per treatment group. Results are expressed in percentage of mortality.

Discussion

IFN-γ, TNF, and IL-1 are involved in the generalized Shwartzman reaction elicited in mice. IFN-γ is the main factor during the priming phase while TNF and IL-1 are the lethal effector molecules induced by the LPS challenge (for review, see 1). The anti-IFN-γ mAbs inhibited the f.p. swelling in mice injected locally with LPS (3) and protected the mice from the lethal reaction (1). Since the monocytes and macrophages appear to be the principal cells involved in mediating the effects of endotoxin (27, 28), it is not clear how LPS can stimulate T and NK cells to release IFN-γ. One hypothesis is that LPS-stimulated macrophages produce IL-1 and IL-6, which in turn activate T cells to release IFN-γ (29). The relatively low incidence of lethality observed in mice primed with these cytokines and challenged with LPS, suggests that IL-1 and IL-6 are cofactors rather than triggering agents in the priming of mice (data not shown).

Recent data showed that LPS can activate macrophages to release IL-12, a cytokine that stimulates T and NK cells to release IFN-γ (9). This finding supports the idea that IL-12 may be the missing link between LPS and IFN-γ in the generalized Shwartzman reaction. Following the same approach as that used for IFN-γ, we conclude that IL-12 and IFN-γ are both involved in the priming phase of generalized Schwartzman reaction. The experiments with anti-IFN-γ and anti-IL-12 antibodies show that the priming effect of IL-12 is mediated by IFN-γ.

IL-12 does not contribute to the lethality of the generalized Shwartzman reaction. The anti-IL-12 antibodies injected together with the intravenous LPS challenge do not rescue the primed animals and IL-12 does not replace the LPS challenge. On the contrary, the LPS primed mice treated with anti-IFN-γ antibodies at the time of the LPS challenge survive. However, IFN-γ does not elicit the lethal reaction when injected instead of the LPS challenge. The protective effect of the anti-IFN-γ antibodies is 10 times less effective than that observed when the same mAbs are given together with the LPS priming. These results show for the first time that IFN-γ contributes to the lethality of the shock reaction but is not a lethal effector molecule. Although evidence points to TNF and IL-1 as the key cytokines leading to the hemodynamic and histologic events of the septic shock (30–33), we never observed a lethal reaction on challenging the mice with either TNF-α (5 µg) or 10⁶ U IL-1 (3.3 µg) after the LPS priming. The injection of TNF-α, IL-1, and IFN-γ mixture can induce an intermediate lethality (33%) in the mice primed with LPS. The coinjection of TNF-α and IL-1 or TNF-α and IFN-γ induces a lethal reaction in mice primed with either IL-12 or IFN-γ. In these conditions, IL-1 and IFN-γ are not lethal. This confirms TNF-α as the main effector of the Shwartzman reaction but that lethality arises in the presence of either IL-1 or IFN-γ. These observations suggest that the f.p. priming injection of LPS induces not only IL-12 and IFN-γ but also antiinflammatory factors. The fact that the priming with high dose of LPS, i.e., 10 µg/mouse, inhibits the lethality induced by the LPS challenge supports this hypothesis (1).

LPS activates many cell types to produce antiinflammatory factors. LPS-activated PMN release enzymes, radicals, and a bactericidal protein called BPI (bactericidal/permeability-increasing protein) (34). This protein competes with lipopolysaccharide binding protein (35) or soluble CD14 and neutralizes LPS so that it cannot activate monocyte/macrophages or injure endothelial cells (36, 37). Upon LPS activation, monocytes produce not only IL-12, TNF-α, and IL-1 but also IL-1 receptor antagonist (IL-1ra), IL-1ra, IL-6 and IL-10 (38–40). Both IL-6 and IL-1ra can also antagonize the production and/or the activity of IL-1 (38, 41). IL-10 inhibits the production of IFN-γ, the proliferation of Th1 cells (42) and downregulates several macrophage functions, including the secretion of IL-1, IL-6, IL-8, IL-10, IL-12, and TNF-α, respiratory burst and MHC class II expression (43, 44). Since the maximal production of IL-10 occurs 24–48 h after LPS activation of macrophages, its putative inhibitory effect in the Shwartzman reaction would coincide with the challenging

phase. Thus, IL-10 could interfere with the activity of the lethal effector cytokines. Recently, Bernhagen (45) reported that the in vivo injection of LPS induces the release of macrophage inhibiting factor (MIF) by pituitary cells. This cytokine is involved in the septic shock but its role is not clear. Hypophysectomised mice are more sensitive to endotoxin, whereas the coinjection of MIF and LPS enhances lethality. Since IL-12 and IFN-γ do not induce MIF and IL-6, such a negative regulatory pathway should not take place in mice primed by IL-12 or IFN-γ. Recent reports suggest that IL-12 and IFN-γ have opposite effects on IL-10 production. IFN-γ inhibits IL-10 production by monocytes (46), whereas increased IL-10 production was observed in mice after daily injection of IL-12 for 4–5 d (47), when its relevance for the onset of the shock reaction is questionable.

In conclusion, our data suggest that a cascade of cytokines induces the generalized Shwartzman reaction. The priming f.p. injection of LPS stimulates the release of IL-12 by the macrophages of the draining lymph nodes and activates the production of IFN-γ by T and/or NK cells. IFN-γ promotes the inflammatory functions of macrophage-like and endothelial cells, and sensitizes them to environmental stimuli. Upon the systemic injection of LPS, all the sensitized cells produce 1TNF and IL-1, that together with the newly synthesized IFN-γ, enhance the adherence, and trigger the procoagulant and proinflammatory activities of these cells. Besides IL-1, IFN-γ seems necessary for the manifestation of the TNF lethality in the generalized Shwartzman reaction.

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