INFECTION AND IMMUNITY, May 1992, p. 1994–2001
0019-9567/92/051994-08$02.00/0
Vol. 60, No. 5

Gamma Interferon Mediates *Propionibacterium acnes*-Induced Hypersensitivity to Lipopolysaccharide in Mice

THOMAS KATSCHINSKI, CHRIS GALANOS, ALEXANDRA COUMBOS, AND MARINA A. FREUDENBERG*
Max-Planck-Institut für Immunbiologie, 7800 Freiburg, Germany

Received 18 December 1991/Accepted 19 February 1992

Pretreatment of lipopolysaccharide (LPS)-responder C57BL/10ScSn mice with killed *Propionibacterium acnes* enhanced tumor necrosis factor alpha (TNF-α) production and lethality in response to a subsequent challenge with LPS. Sensitization to LPS increased with time of pretreatment and reached its maximum after 7 days. Sensitization was paralleled by gamma interferon (IFN-γ) production that was detectable from day 3 onward. In contrast, a similar *P. acnes* pretreatment of LPS-nonresponder C57BL/10ScCr mice had no apparent effect on their high resistance to LPS. Challenge with LPS at any time during the 7-day period after *P. acnes* treatment led to no detectable TNF-α formation and caused no lethal effects. The absence of sensitization in C57BL/10ScCr mice was paralleled by an absence of IFN-γ production. Administration of monoclonal IFN-γ antibodies in C57BL/10ScSn mice up to day 3 of *P. acnes* treatment completely inhibited the overproduction of TNF-α by LPS. Anti-IFN-γ administered later than day 3 had only a partial, although significant, inhibitory effect. Injection of appropriate amounts of anti-IFN-γ also abolished the development of hypersensitivity to the lethal action of LPS. The effect of exogenously administered IFN-γ on LPS sensitivity (e.g., TNF-α production, lethal effects) was studied in LPS-responder and nonresponder mice. Administration of murine recombinant IFN-γ increased the sensitivity of C57BL/10ScSn mice to LPS and established LPS responsiveness in LPS-nonresponder C57BL/10ScCr and C3H/HeJ mice. The data provide evidence that IFN-γ mediates the sensitization towards LPS induced by *P. acnes*.

Bacterial lipopolysaccharides (LPS, endotoxins) are causative agents of a number of pathophysiological reactions seen during infection with gram-negative pathogens. Many of these reactions are elicited by purified LPS in experimental animals (for a review, see reference 30). The interaction of LPS with macrophages and the subsequent formation of endogenous mediators represent essential early steps in the development of LPS lethal toxicity (16, 18, 31, 44). Tumor necrosis factor alpha (TNF-α), a macrophage-derived cytokine inducible by LPS (5, 45), has been recognized as a principal mediator of endotoxin shock. Injection of mice with exogenous TNF-α leads to pathophysiological reactions similar to those seen after LPS administration (36, 38, 60). Antibodies against TNF-α were shown in different experimental models to protect mice from LPS lethality (2, 17, 20, 61).

Susceptibility to LPS of different mouse strains is genetically determined. LPS responsiveness is controlled by a gene locus identified on mouse chromosome 4. It occurs in two allelic forms, lps^n for normal and lps^d for defective responsiveness (51). To date, three lps^d mouse strains, C3H/HeJ, C57BL10/ScCr (Cr), and C57BL/10ScSN (Sn), are known (10, 51, 56, 62). Mice belonging to these strains are highly resistant to all known LPS effects, including its lethal toxicity. Although the defect of lps^d mice concerns the same gene locus, an interesting difference between C3H/HeJ and Cr mice has been identified. This concerns the sensitivity of the two strains towards LPS after infection with *Salmonella typhimurium*. Infected C3H/HeJ mice acquire partial sensitivity to the lethal effects of LPS, whereas Cr mice do not (19). In this respect, C3H/HeJ mice behave like lps^n mice, being sensitized to the lethal effects of LPS by a variety of experimental infections (7, 8, 53, 57, 63). Hypersensitivity is also elicited if, instead of live microorganisms, dead microorganisms are administered in sufficient amounts to the animals (7, 53). Accordingly, lps^n mice and the lps^d C3H/HeJ mice seem to possess similar mechanisms of sensitization to LPS. In contrast, the lps^d Cr mice, depending on the pathogen, show only low (*Plasmodium chabaudi chabaudi*) or no (*S. typhimurium*) sensitivity to LPS after infection. Moreover, Cr mice, in contrast to congenic lps^n Sn mice and lps^d C3H/HeJ mice, exhibit an impaired capacity to produce gamma interferon (IFN-γ) in response to different stimuli, both in vivo and in vitro (19). On the basis of these findings, we asked whether IFN-γ could be involved in the development of hypersensitivity to the lethal effects of LPS.

IFN-γ is produced by T lymphocytes (42, 47, 48) and by natural killer cells (12, 27) in response to specific antigens (52), T-cell mitogens (33), toxic shock syndrome toxin 1 (34), and LPS (4, 37, 43). IFN-γ acts as a macrophage-activating factor. In this function, it enhances antimicrobial activity against some parasites (35), oxidative metabolism (46), and tumor cell-killing activity (13, 49). Furthermore, it increases Fc receptor capacity (14), and induces the expression of Ia antigen on macrophages (6). It has also been shown that IFN-γ increases the ability of macrophages to produce endogenous mediators like TNF-α and interleukin-1 in response to LPS (9, 11, 15, 24). Also, lps^d C3H/HeJ macrophages, after treatment with IFN-γ in culture, acquire the ability to produce TNF-α in response to LPS (3). An increased ability to produce TNF-α after administration of LPS (5, 21) is also a common characteristic of mice sensitized to LPS by infection.

In the present study, the possible involvement of IFN-γ in the sensitization to LPS induced by bacteria was investigated in lps^n (Sn) and lps^d (Cr) mice treated with *Propionibacterium acnes* (1, 5, 21, 25, 26). It was shown that the development of sensitization occurs in parallel with the production of IFN-γ.

* Corresponding author.

MATERIALS AND METHODS

Animals. *lps*^{n} Sn mice and *lps*^{d} Cr and C3H/HeJ mice (of both sexes), bred under specific pathogen-free conditions, were obtained from the breeding stock of the Max-Planck-Institut für Immunbiologie. For lethality tests in the D-galactosamine model (22), 10- to 14-week-old mice were employed; otherwise, 6- to 8-week-old animals were used throughout the study. Mice were challenged with the agents being tested dissolved in pyrogen-free phosphate-buffered saline (pH 7.2) (PBS). Injections were performed intraperitoneally (i.p.) or intravenously (i.v.) into the lateral tail vein.

Serum sampling. Mice were exsanguinated by puncturing the axillary vessels during ether anesthesia. Thereafter, blood samples were incubated for 15 min at 37°C to allow clotting. The serum was separated by centrifugation and stored in aliquots at −70°C until use.

P. acnes. *P. acnes* ATCC 12930 was grown in cooked meat medium (Difco Laboratories, Detroit, Mich.) at 37°C for 4 days without oxygen. Thereafter, the bacteria were washed twice with PBS, heat killed (30 min, 100°C), washed once with distilled water, and lyophilized. For treatment of mice, 500 μg of *P. acnes* in 0.2 ml of PBS per animal was injected i.v.

LPS. The uniform triethylamine salt form of *Salmonella abortus equi* LPS was prepared as described previously (23, 64).

D-Galactosamine. D-Galactosamine–hydrochloride was purchased from C. Roth, Karlsruhe, Germany. For injection, it was dissolved in pyrogen-free PBS.

TNF-α and IFN-γ. Human recombinant TNF-α (specific activity, 6.6×10^{6} U/mg) was a kind gift from Knoll AG, Ludwigshafen, Germany. Murine recombinant TNF-α (specific activity, 1.2×10^{7} U/mg) and murine recombinant IFN-γ (specific activity, 2×10^{7} U/mg) were kindly donated by G. R. Adolf, Bender & Co. GmbH, Vienna, Austria.

Antibodies. Hybridoma cell lines producing rat monoclonal IFN-γ antibodies were kindly donated by S. Landolfo (AN18.17.24; Institute of Microbiology, Medical School, University of Torino, Torino, Italy) (50) and G. L. Spitalny (R46A2; Trudeau Institute, Saranac Lake, N.Y.) (55). The hybridoma cells were cultivated in hydrophobic Teflon bags (Biofolie 2S; DuPont, distributed by Heraeus, Hanau, Germany) by using Iscove's modified Dulbecco's medium (GIBCO Europe Ltd.) containing 10% fetal calf serum at 37°C in a humidified atmosphere containing 8% CO_2. The antibodies were purified from the culture supernatant on an Immuno Pure immobilized protein G column (Pierce, Rockford, Ill.). Subsequently, both antibodies were extensively dialyzed against PBS and sterilized by filtration through a 0.45-μm-pore-size membrane, and protein concentration was determined by the Folin reagent method (40). In this way, a purification factor of 150 was achieved. As a control, rat immunoglobulin G (IgG) purified from normal rat serum by the same procedure was used.

Anti-IFN-γ activity in the antibody preparations was determined by enzyme-linked immunosorbent assay (ELISA) (see below). Possible LPS contamination was measured by the *Limulus* amoebocyte lysate test (59). For animal experiments, the two monoclonal antibodies were always mixed in a ratio of 1:1 (ELISA units). Only preparations with no detectable LPS contamination (<10 pg/mg of protein) were used.

Anti-IFN-γ ELISA. The activity of the antibodies against IFN-γ was assayed by a specific ELISA technique. Briefly, 96-well ELISA microtiter plates (Falcon 3915, ProBind assay plate; Becton Dickinson, Lincoln Park, N.J.) were coated with recombinant IFN-γ (50 μl, 10 μg/ml) in 0.05 M carbonate buffer (pH 9.6). The plates were incubated with 1% bovine serum albumin in PBS (200 μl) for 2 h at 37°C to block the remaining sites. Samples diluted in 1% bovine serum albumin were added, and the plates were incubated for 1 h at 37°C. The plates were washed four times with 0.05% Tween 20 in PBS (pH 7.2) and incubated for 1 h at 37°C with anti-rat IgG-peroxidase conjugate (DAKO GmbH, Hamburg, Germany) diluted 1:2,500 in PBS. After subsequent washing, tetramethylbenzidine was added as the substrate (50 μl) (39), and the plates were kept for 15 min at 37°C in the dark. The reaction was stopped by adding 50 μl of 2 M H_2SO_4 per well. Optical density was measured by a fully automatic ELISA reader workstation (SLT Labinstruments, Crailsheim, Germany) at 450 nm. One-tenth of an ELISA unit of antibody activity is arbitrarily defined as the amount of anti-IFN-γ which corresponds to an optical density of 0.600 in the ELISA described above.

IFN-γ production by spleen cells in vitro. IFN-γ responses in *P. acnes*-treated mice and in controls were determined preferentially by measuring the spontaneous IFN-γ production by spleen cells explanted from the animals. As shown in a previous study (19), this method is very sensitive because it detects ongoing IFN-γ responses that may be too low to be detectable in the circulation. Spleen cells were prepared as previously described (58). The cells (10^{7}/0.5 ml) were cultivated in multidish 24 plastic plates (Nunclon Delta S1; Nunc, Roskilde, Denmark), in Dulbecco modified Eagle medium (GIBCO, Karlsruhe, Germany) for 24 h at 37°C in a humidified atmosphere containing 8% CO_2. After centrifugation, the culture supernatants were collected and stored at −70°C until assayed.

Detection of IFN-γ in culture supernatants. IFN-γ was measured by a specific ELISA technique as described earlier (54).

TNF-α bioassay. The content of TNF-α in individual serum samples was measured in a cytotoxicity test by using a TNF-α-sensitive L929 cell line (kindly provided by L. Old, Memorial Sloan-Kettering Cancer Center, New York, N.Y.), in the presence of actinomycin D as described previously (17). The detection limit of the assay was 8 to 10 pg/ml. Rabbit anti-mouse TNF-α (Genzyme, Boston, Mass.) was used as an inhibitor to test the specificity of the TNF-α bioassay.

Lethality tests. For lethality tests in normal and *P. acnes*-sensitized mice, groups of 3 to 10 animals received different amounts of LPS (0.1 μg to 2,000 μg) i.v. in 0.2 ml of PBS, and the resulting lethality was observed for the next 72 h. For lethality tests in the D-galactosamine-sensitized mice (22), groups of three to six animals received LPS (30 pg to 2 mg) and D-galactosamine (20 mg) as a mixture in 0.2 ml of PBS i.v., and the resulting lethality was scored for the next 20 h.

Statistics. The data shown in the figures are presented with standard deviations. Values were determined by the Student's t test. Values with P values of <0.05 are marked by an asterisk.

RESULTS

Effect of treatment with *P. acnes* on the LPS-induced TNF-α production and lethality in *lps*^{n} and *lps*^{d} mice. Groups of *lps*^{n} Sn and *lps*^{d} Cr mice received killed *P. acnes* (500 μg per mouse) i.v. Untreated groups served as controls. Three and 7 days later, all groups were challenged with LPS i.v., and

TABLE 1. Sensitization of Sn mice to the lethal effects of LPS by *P. acnes*[a]

LPS (μg)	Lethality (no. dead/total)		
	Control	After *P. acnes* administration	
		Day 3	Day 7
100	5/5		
75	1/5		
10	0/5	5/5	
1		0/5	5/5
0.1			5/10
0.01			0/5

[a] Each mouse received 500 μg of *P. acnes* i.v. and, 3 or 7 days later, LPS i.v. Controls received LPS only. Lethality was scored up to 72 h after LPS injection.

the TNF-α present in the serum at 1 h after challenge was measured. Mice not challenged with LPS (*P. acnes* treated or controls) never exhibited TNF-α in serum. After challenge with 0.1 μg of LPS, each of the control Sn mice, not pretreated with *P. acnes*, exhibited, on average, 1.5 ng of TNF-α per ml of serum, whereas control Cr mice, each challenged with either 0.1 or 100 μg of LPS, showed no detectable TNF-α in serum. In *P. acnes*-pretreated Sn mice, the amount of TNF-α produced after challenge with 0.1 μg of LPS increased with time of pretreatment (115 ng/ml of serum when challenged on day 3 and 3,400 ng/ml when challenged on day 7 after pretreatment). In contrast, pretreatment of Cr mice with *P. acnes* did not alter their inability to produce TNF-α in response to LPS. TNF-α was absent at all times, even when 100 μg of LPS was used for challenge.

To estimate the effect of *P. acnes* treatment on the sensitivity of Sn and Cr mice to the lethal effects of LPS, pretreated and control mice of each strain received different amounts of LPS and lethality was scored up to 72 h later. *P. acnes* pretreatment increased the sensitivity of Sn mice to LPS by factors of approximately 10 and 1,000 on days 3 and 7, respectively, as compared to that of the controls (Table 1). *P. acnes*-treated Cr mice, on the other hand, remained LPS resistant, exhibiting no lethality even when challenged with 2 mg of LPS.

IFN-γ production by spleen cells from *P. acnes*-treated mice. Spleen cells from Sn and Cr mice obtained between 1 and 7 days after *P. acnes* treatment were cultured without further stimulus for 24 h along with spleen cells from controls, and the IFN-γ in culture supernatants was measured. Supernatants of control cells were always free of IFN-γ. In cells from *P. acnes*-treated Sn mice, IFN-γ was detectable from day 3 of treatment on in amounts varying between 2 and 50 U/ml. This variation did not seem to be related to the length of *P. acnes* pretreatment. In contrast, at no time did supernatants of cells obtained from *P. acnes*-treated Cr mice exhibit detectable IFN-γ.

Effect of anti-IFN-γ on *P. acnes*-induced sensitization to LPS. (i) TNF-α production. Mice were treated with *P. acnes*. Immediately afterwards and 2, 4, and 6 days later, they received 100 or 300 U of monoclonal anti-IFN-γ antibodies (corresponding to 100 and 300 μg of antibody protein). *P. acnes*-treated mice receiving normal rat IgG (four times, 300 μg) or no further treatment served as controls. On day 7, all mice were challenged with 0.1 μg of LPS, and the level of TNF-α in serum was determined 1 h later.

P. acnes-sensitized mice without further treatment pro-

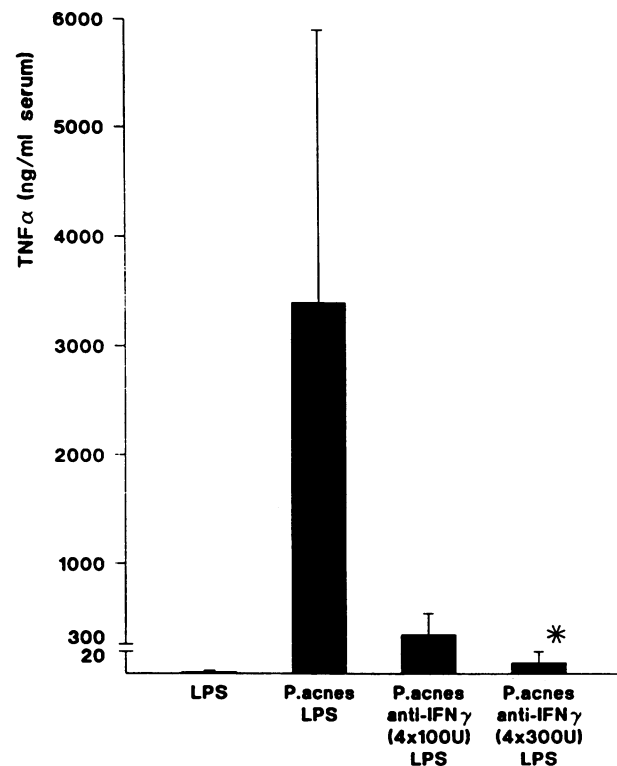

FIG. 1. Effect of anti-IFN-γ on the enhanced TNF-α production by LPS in *P. acnes*-sensitized mice. Each of the Sn mice received 500 μg of *P. acnes* i.v., and the mice were divided into three groups of four animals each. The animals in two groups each received four injections of 100 or 300 U anti-IFN-γ i.p. on days 0, 2, 4, and 6, respectively; the third group remained untreated. On day 7 after *P. acnes* treatment, each animal in all three groups and in an additional group of four normal mice without prior treatment was challenged with 0.1 μg of LPS i.v. TNF-α was measured in serum collected 1 h after challenge. The vertical lines represent standard deviations of mean values. Asterisk, $P < 0.05$.

duced, on average, 3,400 ng of TNF-α per ml of serum in response to 0.1 μg of LPS (Fig. 1). A comparable response (2,500 ± 1,000 ng) was also obtained in the group treated with control IgG (data not shown in Fig. 1). However, mice receiving 100 or 300 U (four times) of anti-IFN-γ exhibited only 1/10 (350 ng/ml) and 1/100 (9.4 ng/ml) as much TNF-α, respectively.

In mice receiving 300 U (four times) of anti-IFN-γ, detectable amounts of free antibody persisted in the serum (average, 20 U/ml) on day 7, while in mice receiving 100 U (four times), anti-IFN-γ activity was no longer detectable by this time.

To determine the time course of IFN-γ action, groups of Sn mice were treated with *P. acnes*. At different times thereafter (0, 24, 48, and 72 h and 4, 5, 6, and 7 days), each mouse in each group was injected once with 400 U of anti-IFN-γ. On day 7, each mouse was challenged with 0.1 μg of LPS, and serum was prepared 1 h later. As shown in Fig. 2, when the antibody was given up to day 3, the TNF-α production, in response to LPS, was inhibited to a level below 13 ng/ml of serum. Administration of anti-IFN-γ at a later time (on days 4, 5, and 6) resulted in a lower inhibition of TNF-α response (30, 69, and 190 ng/ml of serum, respectively). Administration of the antibody on day 7, 2 h before

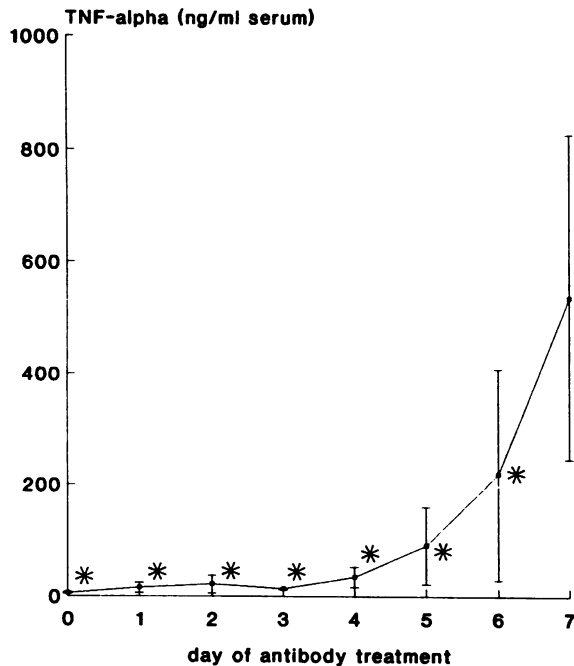

FIG. 2. Effect of anti-IFN-γ administered on different days on the enhanced TNF-α production by LPS in *P. acnes*-sensitized mice. Each of the Sn mice received 500 μg of *P. acnes* i.v. Thereafter, each mouse (in groups of four) received a single injection of anti-IFN-γ (400 U) i.p. on the days indicated. A *P. acnes*-treated group receiving no anti-IFN-γ served as the control. On day 7 after *P. acnes* treatment, each of the mice in all groups was challenged with 0.1 μg of LPS, and TNF-α present in serum collected 1 h after challenge was measured. The control mice produced an average of 1,466 ng of TNF-α per ml of serum. The vertical lines represent standard deviations of mean values. Asterisk, $P < 0.05$.

challenge with LPS, inhibited TNF-α production only to a low extent (592 ng/ml compared to 1,466 ng/ml in controls without antibody treatment). Thus, administration of anti-IFN-γ up to day 3 after *P. acnes* treatment maximally inhibits the enhanced production of TNF-α that is seen on day 7 after LPS challenge.

Administration of anti-IFN-γ on day 3 of *P. acnes* sensitization and subsequent challenge with LPS at different times thereafter revealed that the enhanced production of TNF-α in response to LPS subsides within 24 h of antibody treatment (results not shown).

(ii) **Lethality.** Each of the Sn mice received *P. acnes* and, shortly afterwards, 300 U of monoclonal IFN-γ antibodies i.p. On days 2, 4, and 6, antibody injections were repeated. Each of the control animals was injected in parallel with 300 μg of control IgG four times. On day 7, different amounts of LPS (0.01 to 100 μg) were injected, and the resulting lethality was scored. As shown in Table 2, administration of control antibodies did not inhibit the development of hypersensitivity to LPS. The lethal dose of LPS (40% lethal dose = 0.1 μg) was similar to that seen in *P. acnes*-sensitized mice receiving no antibodies (Table 1). In contrast, administration of anti-IFN-γ markedly inhibited the development of hypersensitivity to LPS (50 μg of LPS being necessary to cause 60% lethality).

It is therefore concluded that anti-IFN-γ antibodies inhibit the development of sensitization to LPS in *P. acnes*-treated mice.

TABLE 2. Effect of IFN-γ antibodies on the *P. acnes*-induced sensitization to LPS lethality[a]

LPS (μg)	Lethality (no. dead/total)		
	Controls	*P. acnes* + IgG	*P. acnes* + MAb[b]
100	5/5		
75	1/5		
50	0/10		6/10
25			0/5
1		5/5	
0.1		2/5	
0.01		0/5	

[a] Each of the Sn mice received 500 μg of *P. acnes* i.v. Thereafter, each of the mice in one group received 300 μg (300 U) of anti-IFN-γ four times, and each of the mice in a second group received 300 μg of control IgG four times i.p., on days 0, 2, 4, and 6 after *P. acnes* treatment. Seven days after *P. acnes* treatment, all mice were challenged with LPS i.v. Normal, untreated Sn mice, injected with LPS only, served as controls. Lethality was scored up to 72 h after LPS injection.

[b] MAb, monoclonal antibody.

Effect of pretreatment with recombinant IFN-γ on TNF-α production. Each of the Sn mice received 10 μg of recombinant murine IFN-γ i.v. At different times thereafter, each group was challenged with 0.1 μg of LPS. At 1 h after challenge, the animals were sacrificed and serum for TNF-γ determination was collected. A group in which each mouse received 0.1 μg of LPS but no IFN-γ served as the control. As shown in Fig. 3, IFN-γ-treated mice invariably produced more TNF-α than the controls. The highest TNF-α levels were obtained when IFN-γ was injected 8 h prior to LPS, in which case an approximately 200-fold increase in TNF-α, compared to that of controls, was seen. With a longer pretreatment time, the enhancing effect of IFN-γ decreased but was still detectable (twofold TNF-α increase) when IFN-γ was injected 24 h before LPS.

A low but reproducibly demonstrable TNF-α response after treatment with IFN-γ (10 μg) was also obtained in lps^d (Cr and C3H/HeJ) mice after challenge with LPS (100 μg per mouse). The TNF-α responses in serum samples of the two strains were comparable (approximately 2 ng/ml). Unlike the case in Sn mice, the time of IFN-γ pretreatment did not seem to be critical in lps^d mice. The administration of IFN-γ between 2 and 8 h prior to LPS resulted in comparable amounts of TNF-α in the serum samples.

The time course of LPS-induced TNF-α production in IFN-γ-pretreated lps^n Sn and lps^d Cr mice is shown in Fig. 4. The timely appearance, duration, and disappearance of TNF-α in serum samples of both strains were similar to those seen usually in responder mice receiving LPS (maximum at 1 h and then decreasing and no longer detectable at 4 h after LPS administration).

Effect of IFN-γ pretreatment on the lethal effects of LPS. Groups of Sn mice (four animals per group), in which each mouse was pretreated with 10 μg of recombinant IFN-γ i.v., or controls were challenged 8 h later with different amounts of LPS (25 to 100 μg per mouse). Lethality was scored over the next 72 h. Administration of 100 μg of LPS caused 100% lethality in control mice. Treatment with 10 μg of IFN-γ increased the sensitivity of mice to LPS, 50 μg of the latter causing 100% lethality. A further increase in susceptibility to the lethal effects of LPS was achieved by two injections of 10 μg of IFN-γ each, administered subcutaneously 8 and 2 h before LPS. Under these conditions, 25 μg of LPS sufficed to cause 100% lethality.

The effect of IFN-γ on the sensitivity of Sn mice to the

TABLE 3. Lethal toxicity of LPS–D-galactosamine in IFN-γ-treated *lps*[n] (C57BL/10ScSn) and *lps*[d] (C57BL/10ScCr, C3H/HeJ) mice[a]

LPS (μg)	Lethality (no. dead/total)					
	IFN-γ treated			Control		
	Sn	Cr	HeJ	Sn	Cr	HeJ
500					0/5	0/5
100		3/3	3/3			
10		4/6	5/6			
1		0/3	0/3	4/4		
0.0003	4/4			3/4		
0.0001	4/4			0/4		
0.00003	0/4					

[a] Each of Sn, Cr, and C3H/HeJ mice was injected with 10 μg of IFN-γ i.v. Control mice remained untreated. At 2 (Cr, C3H/HeJ) or 8 h (Sn) thereafter, they received different amounts of LPS and D-galactosamine (20 mg) as a mixture i.v. All IFN-γ-treated and control mice receiving D-galactosamine or LPS only survived. Lethality was scored up to 24 h after LPS–D-galactosamine injection.

lethal effects of LPS was also investigated in the D-galactosamine sensitization model (22). In this model, treatment of mice with D-galactosamine enhances their susceptibility to the lethal action of LPS by increasing the sensitivity to the lethal mediator TNF-α (17, 20). In this experiment, IFN-γ (10 μg) was administered 8 h before LPS–D-galactosamine challenge (Table 3). Pretreatment with IFN-γ lowered the amount of LPS required for induction of lethality by a factor of 10 as compared to controls receiving LPS-D-galactosamine and no IFN-γ.

The effect of IFN-γ pretreatment on the sensitivity of *lps*[d] C3H/HeJ and Cr mice was investigated only in the D-galactosamine model. Each mouse received 10 μg of IFN-γ and 2 h later was challenged with 20 mg of D-galactosamine and different amounts of LPS (1 to 500 μg) (Table 3). In the controls of both strains, 500 μg of LPS caused no lethality. In contrast, IFN-γ pretreatment rendered both strains considerably more sensitive to LPS, 10-μg amounts of the latter causing 75 and 50% lethality in the C3H/HeJ and Cr mice, respectively.

DISCUSSION

The results of the present study strongly suggest a role for IFN-γ in the *P. acnes*-induced hypersensitivity to LPS. Administration of killed *P. acnes* bacteria to *lps*[n] Sn mice resulted in IFN-γ production and, in parallel, in sensitization of the treated mice to the lethal effects of LPS. As shown in earlier reports (1, 5, 21, 25, 26) and the present study, the LPS hypersensitivity of *P. acnes*-treated mice was evident from the enhanced production of TNF-α upon challenge with LPS and from the higher susceptibility of the treated animals to the lethal activity of LPS. Induction of TNF-α and lethality by LPS were higher on day 7 of *P. acnes* pretreatment than in controls by a factor of approximately 100 to 1,000. Cultures of spleen cells explanted from mice between days 3 and 7 post-*P. acnes* injection spontaneously produced IFN-γ. The production of IFN-γ occurring parallel to the development of sensitization to LPS in *P. acnes*-treated Sn mice is in agreement with previous results obtained in mice sensitized to LPS by two other microorganisms, *S. typhimurium* and *P. chabaudi chabaudi* (19). In contrast, treatment of *lps*[d] Cr mice with *P. acnes* did not lead to detectable amounts of IFN-γ. Furthermore, the treated mice did not produce TNF-α upon LPS challenge and they remained highly resistant to the lethal effects of LPS. The inability of Cr mice to produce IFN-γ after *P. acnes* treatment confirms our previous finding that Cr mice exhibit impaired IFN-γ production (19).

Direct evidence for the participation of IFN-γ in the development of sensitization to LPS was obtained by showing that the overproduction of TNF-α and the enhanced lethality in response to LPS, both characteristics of *P. acnes*-treated mice, could be inhibited by anti-IFN-γ. The inhibition of TNF-α overproduction by IFN-γ antibodies was more effective when the antibodies were administered during the first 3 days after *P. acnes* treatment. Administration of anti-IFN-γ after day 3 of *P. acnes* treatment was less effective; however, some protective effect was still evident even when anti-IFN-γ was administered on day 7, 2 h before LPS challenge.

In agreement with results of a previous study (28), the present results show that normal Sn mice (without *P. acnes*), administered murine recombinant IFN-γ (10 μg per mouse), produce significantly more TNF-α upon challenge with LPS. Increased TNF-α production became evident soon after IFN-γ treatment, was highest after 8 h, and disappeared 24 h after IFN-γ administration. The in vivo kinetics of IFN-γ sensitization corresponds well to the kinetics observed by Luedke and Cerami (41) in vitro after the addition of IFN-γ to macrophage cultures. Sn mice treated with IFN-γ 8 h before LPS challenge also became more sensitive to the

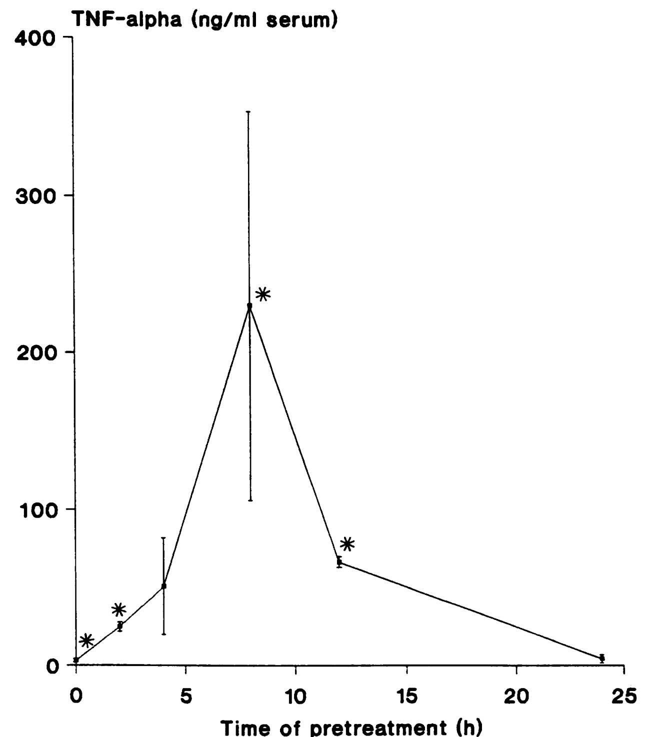

FIG. 3. Enhancement of LPS-induced TNF-α production by pretreatment with IFN-γ. Each of the Sn mice received 10 μg of murine recombinant IFN-γ i.v. At different times thereafter, each mouse (in groups of three), was challenged with 0.1 μg of LPS i.v., and the TNF-α present in the serum collected 1 h later was measured. Control mice receiving LPS but not IFN-γ produced an average of 1.17 ng/ml of serum. The vertical lines represent standard deviations of mean values. Asterisk, $P < 0.05$.

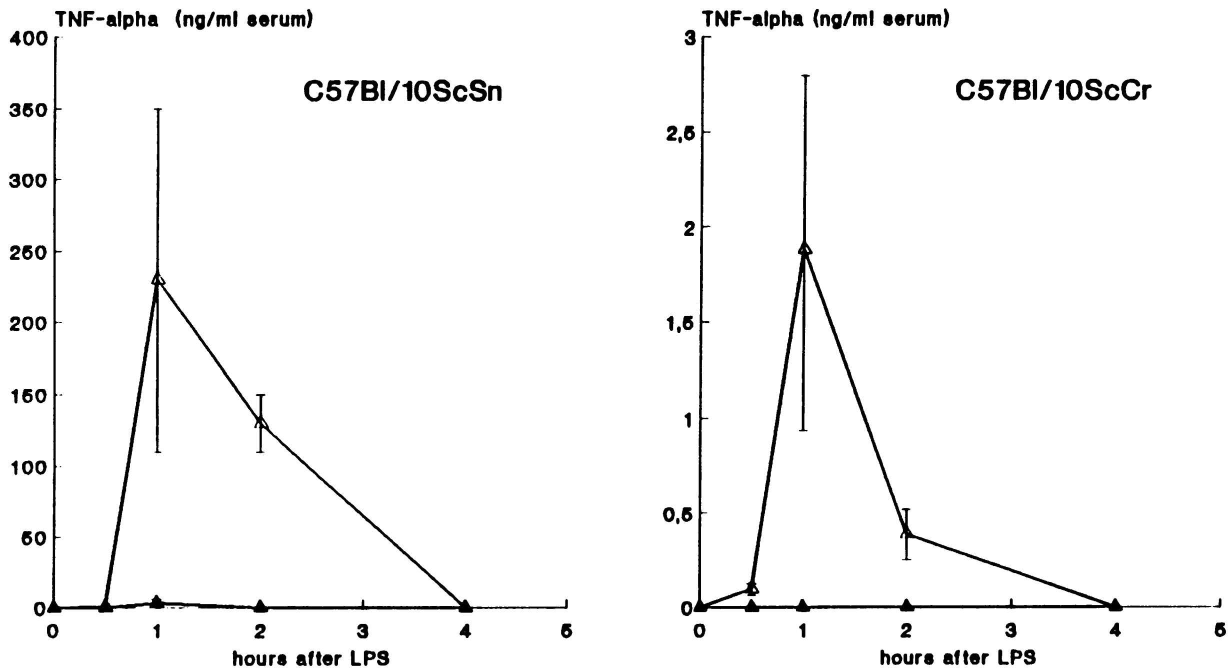

FIG. 4. Time course of TNF-α production by LPS in *lps*^{n} and *lps*^{d} mice pretreated with IFN-γ. Each of the Sn and Cr mice was injected with 10 μg of murine recombinant IFN-γ. Eight hours later, the animals were challenged with LPS, with each of the Sn mice receiving 0.1 μg and each of the Cr mice receiving 100 μg. Serum for TNF-α estimation was collected at different times after challenge. Each point on the curve represents a mean of the results from three individual animals with standard deviation (vertical lines).

lethal effects of LPS as tested in control mice and in mice sensitized to LPS by D-galactosamine. *lps*^{d} Cr and C3H/HeJ mice, although differing in their IFN-γ productions (Cr, defective production; C3H/HeJ, normal production), exhibited very comparable responsiveness to IFN-γ. Mice of both strains acquired a partial responsiveness to LPS when administered IFN-γ and exhibited a low level of circulating TNF-α (approximately 2 ng/ml) after challenge with a high dose (100 μg per mouse) of LPS. In comparison, each of the control Sn mice exhibit a few nanograms of TNF-α per ml of serum and each of the IFN-γ-treated Sn mice exhibit several thousand nanograms per ml when challenged with 0.1 μg of LPS. It is interesting that, unlike in *lps*^{n} Sn mice, in which maximum sensitization was seen 8 h after IFN-γ treatment, in *lps*^{d} mice there was no difference in the degree of sensitization whether IFN-γ was injected at 2 or at 8 h prior to LPS. By using the D-galactosamine sensitization model, it could be shown that pretreatment with IFN-γ also enhanced the sensitivity of both *lps*^{d} mouse strains towards a lethal combination of LPS and D-galactosamine.

Recently, Hogan and Vogel (32) pointed out that the LPS response seen in C3H/HeJ mice that had been treated with IFN-γ might be explained by a sensitization to an unidentified bacterial component rather than a reversal of LPS nonresponsiveness. Experimental support for this was presented. The presence of a non-LPS component(s) in gram-negative bacteria with LPS-like activities, such as induction of TNF-α and lethality, has been recently demonstrated with C3H/HeJ and Cr mice (17). Furthermore, the sensitizing effect of IFN-γ is not restricted to the action of LPS. We have observed that exogenously administered murine recombinant IFN-γ increases the sensitivity of *lps*^{n} Sn and *lps*^{d} Cr mice towards the TNF-α-inducing activity of *Staphylococcus aureus* (15a). The LPS of *S. abortus equi* used in the present study was extensively purified. Protein, lipid, glycan, and nucleic acid contaminants were not detectable in the final product. The preparation also showed no biological activity in a number of biological assays in *lps*^{d} mice. However, a minute contamination of a natural bacterial product can never be excluded with 100% certainty. More experimental work is required to obtain a definite explanation of the nature of LPS resistance of *lps*^{d} mice.

In this study, we could also confirm previous findings (28, 29) that administration of anti-IFN-γ has a low protective effect against LPS toxicity in normal *lps*^{n} mice (not treated with *P. acnes*) (data not shown). Normal mice, treated 2 h before or 10 min after LPS challenge with antibodies, were more resistant to the lethal effects of LPS by a factor of 2. IFN-γ, therefore, apart from its prominent role in sensitizing to LPS, also seems to participate in the actual mechanisms of toxicity.

Our results show that IFN-γ is a principal mediator involved in the development of hypersensitivity to LPS in mice treated with *P. acnes* and very probably in the hypersensitivity of mice infected by different pathogenic microorganisms. Treatment of human monocytes with human recombinant IFN-γ in vitro (15, 24) or administration of IFN-γ in cancer patients (41a) were shown to increase the LPS-induced cytokine response. IFN-γ may be, therefore, a possible mediator of LPS hypersensitivity in infected patients as well, and its production may lower the threshold of the development of endotoxin shock.

ACKNOWLEDGMENTS

We thank H. Stübig and C. Steidle for their excellent technical assistance.

This study was supported in part by the Deutsche Forschungsge-

meinschaft through SFB 154 and by the Bundesminister für Forschung und Technologie (grant 01 Ki 8809).

REFERENCES

1. **Berendt, M. J., M. F. Newborg, and R. J. North.** 1980. Increased toxicity of endotoxin for tumor-bearing mice and mice responding to bacterial pathogens: macrophage activation as a common denominator. Infect. Immun. **28:**645–647.
2. **Beutler, B., I. W. Milsark, and A. C. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229:**869–871.
3. **Beutler, B., V. Tkacenko, I. Milsark, N. Krochin, and A. Cerami.** 1986. Effect of γ-interferon on cachectin expression by mononuclear phagocytes. Reversal of the lps^d (endotoxin resistance) phenotype. J. Exp. Med. **164:**1791–1796.
4. **Blanchard, K., J. Y. Djeu, T. W. Klein, H. Friedman, and W. E. Stewart II.** 1986. Interferon-γ induction by lipopolysaccharide: dependence on interleukin 2 and macrophages. J. Immunol. **136:**963–970.
5. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72:**3666–3670.
6. **Chang, R. J., and S. H. Lee.** 1986. Effects of interferon-γ and tumor necrosis factor-α in the expression of an Ia antigen on a murine macrophage cell line. J. Immunol. **137:**2853–2856.
7. **Chedid, L., and M. Parant.** 1971. Role of hypersensitivity and tolerance in reactions to endotoxin, p. 415–459. *In* S. Kadis, G. Weinbaum, and S. J. Ajl (ed.), Microbial toxins, vol. 5, Academic Press, Inc., New York.
8. **Clark, I. A.** 1978. Does endotoxin cause both the disease and parasite death in acute malaria and babesiosis? Lancet **i:**75–77.
9. **Collart, M. A., D. Belin, J. D. Vassalli, S. De Kossodo, and P. Vassalli.** 1986. γ-Interferon enhances macrophage transcription of the tumor necrosis factor-cachectin, interleukin-1, and urokinase genes, which are controlled by short-lived repressors. J. Exp. Med. **164:**2113–2118.
10. **Coutinho, A., and T. Meo.** 1978. Genetic basis for unresponsiveness to lipopolysaccharide in C57BL/10 Cr mice. Immunogenetics **7:**17–24.
11. **Danis, V. A., G. M. Franic, D. A. Rathjen, and T. M. Brooks.** 1991. Effects of granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and IL-6 on the production of immunoreactive IL-1 and TNF-α by human macrophages. J. Clin. Exp. Immunol. **85:**143–150.
12. **Djeu, J. Y., N. Stocks, K. Zoon, G. J. Stanton, T. Timonen, and R. B. Herberman.** 1982. Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes viruses. J. Exp. Med. **156:**1222–1234.
13. **Esparza, I., D. Männel, A. Ruppel, W. Falk, and P. H. Krammer.** 1987. Interferon-γ and lymphotoxin or tumor necrosis factor act synergistically to induce macrophage killing of tumor cells and schistosomula of *Schistosoma mansoni*. J. Exp. Med. **166:**589–594.
14. **Fertsch, D., and S. N. Vogel.** 1984. Recombinant interferons increase macrophage Fc receptor capacity. J. Immunol. **132:**2436–2439.
15. **Fomsgaard, A., M. A. Freudenberg, K. Bendtzen, and C. Galanos.** 1990. Quantitation and biological activities of native tumor necrosis factor from LPS-stimulated human monocytes. APMIS **98:**529–534.

15a. **Freudenberg, M. A.** Unpublished data.

16. **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.
17. **Freudenberg, M. A., and C. Galanos.** 1991. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. Infect. Immun. **59:**2110–2113.
18. **Freudenberg, M. A., D. Keppler, and C. Galanos.** 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. **59:**891–895.
19. **Freudenberg, M. A., Y. Kumazawa, S. Meding, J. Langhorne, and C. Galanos.** 1991. Gamma interferon production in endotoxin-responder and -nonresponder mice during infection. Infect. Immun. **59:**3484–3491.
20. **Galanos, C., and M. A. Freudenberg.** 1990. Tumor necrosis factor mediates endotoxin shock: the protective effects of antibodies and cortisone, p. 187–193. *In* B. Bonavida and G. Granger (ed.), Tumor necrosis factor: structure, mechanism of action, role in disease and therapy. S. Karger, Basel.
21. **Galanos, C., M. A. Freudenberg, A. Coumbos, M. Matsuura, V. Lehmann, 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. International Conference on Tumor Necrosis Factor and Related Cytotoxins, Heidelberg 1987. S. Karger, Basel.
22. **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.
23. **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.
24. **Gifford, G. E., and M. L. Lohmann-Matthes.** 1987. Gamma-interferon priming of mouse and human macrophages for induction of tumor necrosis factor by bacterial lipopolysaccharides. J. Natl. Cancer Inst. **78:**121–124.
25. **Green, S., A. Dobrjansky, M. A. Chiasson, E. Carswell, M. K. Schwartz, and L. J. Old.** 1977. Corynebacterium parvum as the priming agent in the production of tumor necrosis factor in the mouse. J. Natl. Cancer Inst. **59:**1519–1522.
26. **Halpern, B. N., A.-R. Prévot, G. Biozzi, C. Stiffel, D. Mouton, J. C. Morard, Y. Bouthillier, and C. Decreusefond.** 1964. Stimulation de l'activité phagocytaire du système réticuloendothélial provoquée par *Corynebacterium parvum*. RES J. Reticuloendothel. Soc. **1:**77–96.
27. **Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai.** 1983. Natural killer (NK) cells as a responder to interleukin 2 (IL-2). II. IL-2-induced interferon-γ production. J. Immunol. **130:**988–992.
28. **Heinzel, F. P.** 1990. The role of IFN-γ in the pathology of experimental endotoxemia. J. Immunol. **145:**2920–2924.
29. **Heremans, H., J. Van Damme, C. Dillen, R. Dijkmans, and A. Billau.** 1990. Interferon-γ, a mediator of lethal lipopolysaccharide-induced Shwartzman-like shock reactions in mice. J. Exp. Med. **171:**1853–1869.
30. **Hinshaw, L. B. (ed.).** 1985. *In* Handbook of endotoxin. vol. 2. Pathophysiology of endotoxin. 1985. Elsevier, Amsterdam.
31. **Hinshaw, L. B.** 1990. Pathophysiology of endotoxin action: an overview, p. 419–426. *In* A. Nowotny, J. J. Spitzer, and E. J. Ziegler (ed.), Endotoxin research series, vol. 1. Cellular and molecular aspects of endotoxin reactions. Experta Medica, Amsterdam.
32. **Hogan, M. M., and S. N. Vogel.** 1988. Production of tumor necrosis factor by rIFN-γ-primed C3H/HeJ (*lps*^{d}) macrophages requires the presence of lipid A-associated proteins. J. Immunol. **141:**4196–4202.
33. **Johnson, H. M., G. J. Stanton, and S. Baron.** 1977. Relative ability for mitogens to stimulate production of interferon by lymphoid cells and to induce suppression of the *in vitro* immune response. Proc. Soc. Exp. Biol. Med. **154:**138–141.
34. **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.
35. **Kayaga, K., K. Watanabe, and Y. Fukazawa.** 1989. Capacity of recombinant gamma interferon to activate macrophages for *Salmonella*-killing activity. Infect. Immun. **57:**609–615.
36. **Kettelhut, I. C., W. Fiers, and A. L. Goldberg.** 1987. The toxic

effects of tumor necrosis factor *in vivo* and their prevention by cyclooxygenase inhibitors. Proc. Natl. Acad. Sci. USA **84:** 4273–4277.

37. **Le, J., J.-X. Lin, D. Henriksen-DeStefano, and J. Vilček.** 1986. Bacterial lipopolysaccharide-induced interferon-γ production: roles of interleukin 1 and interleukin 2. J. Immunol. **136:**4525–4530.
38. **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.
39. **Liem, H. H., F. Cardenas, M. Tavassoli, M. B. Poh-Fitzpatrick, and U. Müller-Eberhard.** 1979. Quantitative determination of hemoglobin and cytochemical staining for peroxydase using 3,3′,5,5′-tetramethylbenzidine dihydrochloride, a safe substitute for benzidine. Anal. Biochem. **98:**388–395.
40. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
41. **Luedke, C. E., and A. Cerami.** 1990. Interferon-γ overcomes glucocorticoid suppression of cachectin/tumor necrosis factor biosynthesis by murine macrophages. J. Clin. Invest. **86:**1234–1240.

41a. **Mackensen, A., C. Galanos, and R. Engelhardt.** Unpublished data.

42. **Marcucci, F., M. Waller, H. Kirchner, and P. Krammer.** 1981. Production of immune interferon (IFN-γ) by murine T cell clones from long-term cultures. Nature (London) **291:**79–81.
43. **Matsumura, H., and M. Nakano.** 1988. Endotoxin-induced interferon-γ production in culture cells derived from BCG-infected C3H/HeJ mice. J. Immunol. **140:**494–500.
44. **Michalek, S. M., R. N. Moore, J. R. McGhee, D. L. Rosenstreich, and S. E. Mergenhagen.** 1980. The primary role of lymphoreticular cells in the mediation of host responses to bacterial endotoxin. J. Infect. Dis. **141:**55–63.
45. **Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. Owyer, D. A. Dinarello, A. Cerami, S. M. Wolff, and D. W. Wilmore.** 1988. Detection of circulating tumor necrosis factor after endotoxin administration. N. Engl. J. Med. **318:**1481–1486.
46. **Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin.** 1983. Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. **158:**670–689.
47. **Nathan, I., J. E. Groopman, S. G. Quan, N. Bersch, and D. W. Glode.** 1981. Immune (γ) interferon produced by a human T-lymphoblast cell line. Nature (London) **292:**842–844.
48. **O'Garra, A.** 1989. Interleukins and the immune system. Lancet **ii:**1003–1005.
49. **Pace, J. L., S. W. Russell, B. A. Torres, H. M. Johnson, and P. W. Gray.** 1983. Recombinant mouse γ interferon induces the priming step in macrophage activation for tumor cell killing. J. Immunol. **130:**2011–2013.
50. **Prat, M., G. Gribaudo, P. M. Comoglio, G. Cavalio, and S. Landolfo.** 1984. Monoclonal antibodies against murine γ-interferon. Proc. Natl. Acad. Sci. USA **81:**4515–4519.
51. **Rosenstreich, D. L.** 1985. Genetic control of endotoxin response: C3H/HeJ mice, p. 82–122. *In* R. A. Proctor and L. J. Berry (ed.), Handbook of endotoxin, vol. 4. Elsevier, Amsterdam.
52. **Salvin, S. B., J. S. Youngner, and W. H. Lederer.** 1973. Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. Infect. Immun. **7:**68–75.
53. **Schramek, S., J. Kazar, Z. Sekeyova, M. A. Freudenberg, and C. Galanos.** 1984. Induction of hyperreactivity to endotoxin in mice by *Coxiella burnetii*. Infect. Immun. **45:**713–717.
54. **Slade, S. J., and J. Langhorne.** 1989. Production of interferon-gamma during infection of mice with *Plasmodium chabaudi chabaudi*. Immunobiology **179:**353–365.
55. **Spitalny, G. L., and E. A. Havell.** 1984. Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. J. Exp. Med. **159:**1560–1565.
56. **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.
57. **Suter, E., G. E. Ullman, and R. G. Hoffmann.** 1958. Sensitivity of mice to endotoxin after vaccination with BCG (bacillus Calmette-Guérin). Proc. Soc. Exp. Biol. Med. **99:**167–169.
58. **Tanamoto, K., C. Galanos, O. Lüderitz, S. Kusumoto, and T. Shiba.** 1984. Mitogenic activities of synthetic lipid A analogs and supression of mitogenicity of lipid A. Infect. Immun. **44:**427–433.
59. **Tanamoto, K., U. Zähringer, G. R. McKenzie, C. Galanos, E. T. 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.
60. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, J. D. Albert, T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234:** 470–474.
61. **Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami.** 1987. Anticachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature (London) **330:**662–664.
62. **Vogel, S. N., C. T. Hansen, and D. L. Rosenstreich.** 1979. Characterization of a congenitally LPS-resistant, athymic mouse strain. J. Immunol. **122:**619–622.
63. **Vogel, S. N., R. N. Moore, J. D. Sipe, and D. L. Rosenstreich.** 1980. BCG-induced enhancement of endotoxin sensitivity in C3H/HeJ mice. I. *In vivo* studies. J. Immunol. **124:**2004–2009.
64. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. Teil B **7:**148–155.