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Septic shock is a major cause of mortality in neonates. The hypothesis was tested that neonatal age is associated with altered sensitivity to shock-inducing bacterial products or proinflammatory cytokines (or both). Mice of different ages were inoculated with various doses of lipopolysaccharide (LPS), superantigenic staphylococcal enterotoxin B (SEB), or recombinant tumor necrosis factor-α (rTNF-α), alone or in combination with the sensitizing agent D-galactosamine. Neonatal mice were markedly more susceptible to LPS-induced lethality but more resistant to SEB than were adults ($P < .05$). Mice of different ages did not differ, however, in their sensitivity to lethal activities of rTNF-α. Neonatal susceptibility to LPS and SEB correlated directly with plasma TNF-α but not IFN-γ levels, which was confirmed by TNF-α and IFN-γ blockade experiments. These data document marked age-related differences in the pathophysiology of septic shock and suggest that IFN-γ is not an obligatory mediator of either LPS- or SEB-induced lethality in neonates.

Neonatal septic shock differs clinically from adult septic shock. Mortality is higher in neonates (50%–70%), as is the incidence of permanent physical disabilities [1]. In addition, neonates have often atypical manifestations, such as thermal instability, lack of febrile response, and higher incidence of respiratory insufficiency [2]. Hemodynamic features of lipopolysaccharide (LPS)-induced shock in young dogs [3] and piglets [4] include a lack of early systemic hypotension and right ventricular failure.

Many of the manifestations of septic shock have been related to high levels of circulating cytokines and other inflammatory mediators released on interaction of host cells with bacterial products. There are some indications that neonates may have altered cytokine responses. Stimulated T cells from human newborns produced significantly less interferon-γ (IFN-γ) than did those of adults but equal amounts of interleukin-2 (IL-2) [5–7]. T cells from neonatal mice produced minimal IL-2 and IFN-γ but high levels of IL-4 in response to primary stimulation in vitro with anti-CD3 antibody [8].

Studies investigating differences between human neonates and adults in the production of tumor necrosis factor-α (TNF-α), IL-1, or IL-6 have often yielded contradictory results. TNF production by leukocytes in response to LPS from gram-negative bacteria was found to be reduced [9], normal [10, 11], or increased [12] in term neonates compared with adults. Cells from term neonates also showed decreased or normal IL-1 [13–17] and IL-6 [18–20] production. TNF and IL-6 responses were found to be decreased in premature babies [11, 19].

This study was undertaken to test the hypothesis that neonatal age is associated with altered sensitivity to shock-inducing bacterial products or endogenous cytokine mediators (or both). In view of the conflicting results obtained with in vitro studies, we used murine models to assess age-dependent mortality and circulating cytokine levels. LPS and staphylococcal enterotoxin B (SEB) were selected as shock-inducing agents. LPS is considered the main component responsible for mediator production in sepsis caused by gram-negative bacteria, the most frequent cause of systemic infections in the neonate. SEB is a member of a family of superantigenic exotoxins produced by several species of gram-positive bacteria. In contrast to LPS, which acts predominantly on macrophages [21], SEB and other superantigenic exotoxins induce shock by stimulating mainly T cells [22].

Materials and Methods

Mice. BALB/c mice of different ages were used. Parental mice were obtained from Harlan Nossan (Milan, Italy) and housed in the animal facilities of the Institute of Microbiology of the University of Messina. Periodic examinations showed that the colony was free from naturally occurring infections. Females from timed matings were monitored closely and the date of delivery recorded. Adults were defined as 8- to 9-week-old mice of both sexes.

Lethality test. Suckling pups from each litter were randomly assigned to experimental groups, marked, and kept with the mother until completion of the experiments. Mice were weighed and injected subcutaneously with various doses of *Salmonella enteritidis* LPS (Sigma Chimica, Milan, Italy), SEB (Sigma), mouse recombi-

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All experiments described herein were approved by the competent local authorities. All procedures were in agreement with NIH guidelines for the handling of laboratory animals.

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nant (r) TNF-α, or mouse rIFN-γ (both from Genzyme, Cinisello Balsamo, Italy). Where indicated, mice were inoculated subcutaneously with 350 mg/kg D-galactosamine (D-gal) at the same time as LPS, SEB, or rTNF-α challenge. All materials were dissolved and diluted in pyrogen-free PBS (0.01 *M* phosphate, 0.15 *M* NaCl, pH 7.2). Injection volumes were 25, 50, 100, and 250 μL, respectively, in mice aged 1, 8, and 15 days and adults. Lethality was recorded every 12 h for 4 days. Moribund animals were euthanized by ether inhalation.

To assess the protective effects of TNF-α or IFN-γ blockade, animals were inoculated subcutaneously with anti–TNF-α rabbit serum or anti–IFN-γ hamster monoclonal antibody (both from Genzyme) at 4 h before challenge. The neutralizing activities of these preparations were determined in preliminary tests. One unit of anti–TNF-α was defined as the amount of antibody giving 50% neutralization of the cytotoxic effects of 10 U of mouse rTNF-α on WEHI 164 clone 13 cells [23]. One unit of anti–IFN-γ was defined as the amount of antibody giving 50% neutralization of the antiviral effects of 10 U of mouse rIFN-γ on L929 cells infected with vesicular stomatitis virus. All reagents used were free from endotoxin, as determined by the limulus amebocyte lysate assay (Sigma).

Cytokine measurements. To measure circulating cytokine levels, groups of 5–10 animals were sacrificed by decapitation under ether anesthesia at different times after injection with LPS and SEB. Mixed venous-arterial blood was collected in heparinized containers and centrifuged. Pooled plasma from groups of 5–10 animals was stored at −70°C until assayed for cytokine concentrations.

TNF-α activity was quantitated exactly as described [24], by use of a cytotoxicity assay, and was expressed in units per milliliter, 1 U being defined as the amount of cytokine inducing 50% lysis of WEHI 164 clone 13 cells [23]. Seven serial 2-fold dilutions (final dilutions, 1:16 to 1:1024) were tested in duplicate for each sample. The assay was calibrated with murine rTNF-α as a standard. In selected plasma pools, TNF activity was neutralized with rabbit anti–TNF-α or anti–TNF-β serum (both from Genzyme). These studies indicated that TNF bioactivity in plasma from neonates or adults challenged with LPS was entirely due to TNF-α. TNF bioactivity in plasma from either adults or pups injected with SEB was neutralized by 65%–75% with anti–TNF-α and by 35%–45% with anti–TNF-β.

IL-2, IL-4, IL-6, IFN-γ, and IL-1β were measured by use of mouse-specific commercial antigen-capture ELISAs, according to manufacturer's instructions. IL-2, IL-4, and IL-6 kits were gifts from Bender MedSystems (Vienna). IFN-γ and IL-1β kits were purchased from Genzyme. Plasma samples were diluted 1:2 (IL-2, IL-4, IFN-γ, and IL-1β measurements) or 1:100 (IL-6). When absorbance values exceeded the linear portion of the standard curve, samples were diluted further and reassayed. The lower limits of detection, calculated by use of the standard curve, were 30 pg/mL for IL-2, 20 pg/mL for IL-6, 30 pg/mL for IL-1β, 150 pg/mL for IFN-γ, and 25 pg/mL for IL-4. Since samples were diluted before the assays, the actual lower limits of detection were calculated by multiplying the standard curve lower limit by the dilution factors.

Data expression and statistical analysis. Cytokine levels are expressed as means ± SDs of three independent observations, each conducted in duplicate on a different plasma pool. To calculate mean values, results below the detection level were assigned a theoretical value of half of the detection level. Differences in plasma cytokine levels were assessed by one-way analysis of variance and Student-Newman-Keuls test. Differences in lethality were analyzed with Fisher's exact test. With both tests, differences were considered significant at $P < .05$.

Results

Age-related mortality to LPS. Initial experiments were done to determine if mouse pups differed from adult animals in their sensitivity to LPS-induced lethality. Mice of different ages were inoculated with various doses of LPS alone or in combination with the sensitizing agent D-gal. The latter agent has been extensively used to overcome the natural resistance of mice to LPS or SEB [21, 22]. Table 1 shows that 50 mg/kg LPS induced 100% and 71% lethalities, respectively, in 1- and 8-day-old unsensitized pups. In contrast, the same dose did not produce any lethality in 15-day-old or adult animals. Eight-day-old mice exhibited slightly but significantly lower lethality to LPS relative to 1-day-old pups ($P < .05$ with 20 mg/kg LPS). Estimated LD_{50}s were 12, 38, 122, and 139 mg/kg, respectively, for 1-, 8-, and 15-day-old and adult mice. D-gal increased to a similar extent the sensitivity of either neonatal or adult animals (table 1). Estimated LD_{50} values for LPS in D-gal–sensitized mice were 0.2 and 4.5 mg/kg, respectively, in 1-day-old and adult animals. Therefore, the

Table 1. Lethal toxicity of lipopolysaccharide (LPS) in mice of different ages.

Age	D-galactosamine (350 mg/kg)	LPS (mg/kg)	Lethality (dead/total)
1 day	–	1	0/14
	–	10	6/14
	–	20	10/14
	–	50	14/14*
8 days	–	20	3/14
	–	50	10/14*
	–	100	14/14
15 days	–	50	0/14
	–	75	2/14
	–	150	10/14
	–	300	14/14
Adult	–	50	0/14
	–	75	1/14
	–	150	8/14
	–	300	14/14
1 day	+	0.01	0/14
	+	0.1	6/14*
	+	1	13/14*
Adult	+	0.1	0/14
	+	1	3/14
	+	10	13/14

NOTE. Data are cumulative results of 3–5 experiments.
* $P < .05$ compared with adults.

susceptibility to the lethal activities of LPS was >10-fold higher in 1-day-old pups than in adults, both in the presence and in the absence of sensitization with D-gal. LPS-induced lethality showed a prolonged course in neonatal pups. The majority of adult mice died within 24 h, while 1-day-old pups died in 24–72 h (not shown).

rTNF toxicity in neonatal mice. rTNF can reproduce most of the pathophysiologic changes of LPS-induced shock alone or in combination with other proinflammatory cytokines such as IFN-γ. Therefore it was of interest to ascertain if the increased susceptibility of neonates to LPS could be accounted for by differences in sensitivity to endogenous TNF. Table 2 shows that this was unlikely, since 5×10^6 U/kg rTNF-α induced similar mortality in 1-day-old and adult animals sensitized with D-gal. A mixture of rTNF-α (10^6 U/kg) and rIFN-γ (0.5×10^6 U/kg) also induced similar lethality in sensitized adult and neonatal mice. Thus, rTNF-α–induced lethality was at variance with LPS lethality. rTNF-α was able to reproduce in neonatal pups the same changes observed with LPS, including the prolonged course of lethality.

SEB-induced lethality. To determine if other shock-inducing bacterial toxins, in addition to LPS, also caused increased lethality in neonates, the effects of SEB were compared in mice of different ages. This agent was selected because, unlike LPS, it can produce cytokine-mediated toxicity by stimulating predominantly T lymphocytes rather than macrophages [22]. In addition, SEB may play a pathogenic role in infections by enterotoxin-producing *Staphylococcus aureus* isolates, which frequently cause infections in both adults and children [25]. SEB alone was insufficient to induce lethality at doses as high as 200 mg/kg in either neonates or adults (table 3). However, in the presence of D-gal, 5 mg/kg was sufficient to induce lethality in 100% of the 15-day-old or adult animals. In contrast, doses as high as 20 mg/kg did not produce any lethality in 1- or 8-day-old sensitized mice. Estimated LD_{50} values for SEB in D-gal–sensitized mice were 46.0, 35.2, 1.5, and 1.0 mg/kg, respectively, in 1-, 8-, and 15-day-old and adult animals. These studies indicated that 45-fold-higher SEB doses were needed to induce comparable lethality in neonatal mice relative to adults. Again, SEB-induced lethality showed a prolonged course in neonatal mice, but not in adults, paralleling similar changes observed after administration of rTNF-α or LPS.

Table 2. Recombinant tumor necrosis factor-α (rTNF-α) lethality in D-galactosamine–sensitized mice.

Age	Treatment (U/kg × 10^6)		Lethality (dead/total)
	rTNF-α	Recombinant interferon-γ	
1 day	1.0	—	0/6
	5.0	—	4/6
	1.0	0.5	4/6
Adult	1.0	—	0/4
	5.0	—	4/6
	1.0	0.5	5/6

NOTE. Data are cumulative results of 3–5 experiments. Animals were injected with D-galactosamine at same time of injection with recombinant cytokines.

Table 3. Lethal toxicity of staphylococcal enterotoxin B (SEB) in mice of different ages.

Age	D-galactosamine (350 mg/kg)	SEB (mg/kg)	Lethality (dead/total)
1 day	−	10	0/14
	−	200	0/14
Adult	−	10	0/14
	−	200	0/14
1 day	+	10	0/14*
	+	20	0/14
	+	40	6/14
	+	80	13/14
	+	100	14/14
8 days	+	20	1/14
	+	40	9/14
	+	80	14/14
15 days	+	0.1	0/14
	+	1	6/14
	+	5	13/14
	+	10	14/14
Adult	+	0.1	0/14
	+	1	7/14
	+	5	14/14
	+	10	14/14

NOTE. Data are cumulative results of 3–5 experiments.
* $P < .05$ compared with adults.

LPS-induced cytokine production. The above experiments indicated that the increased mortality of neonates to LPS was specific and did not reflect a hypersusceptibility to rTNF-α. Therefore, in further experiments we tested the hypothesis that age-dependent lethality was related to altered cytokine production. Mice were injected with 20 mg/kg LPS, and cytokine levels were measured in plasma samples obtained at different times after challenge.

Significant plasma TNF elevations over baseline (uninjected or 0 h controls) were detected in all age groups at 1, 2, and 3 h after challenge and returned to basal values thereafter (figure 1). The most striking age-related differences were significantly higher ($P < .05$) TNF production in the younger age groups (643 ± 119, 288 ± 106, 53 ± 16, and 24 ± 12 U/mL, respectively, in 1-, 8-, and 15-day-old pups and adults at 2 h after LPS). Differences in kinetics were also apparent. TNF bioactivity reached peak values later but persisted for longer times in 1- and 8-day-old animals compared with the older age groups.

The appearance of IL-1β was also delayed in younger animals compared with adults (figure 2). In addition, IL-1β levels were consistently lower at 2, 3, and 4 h after challenge in 1- and 8-day-old mice compared with adults ($P < .05$).

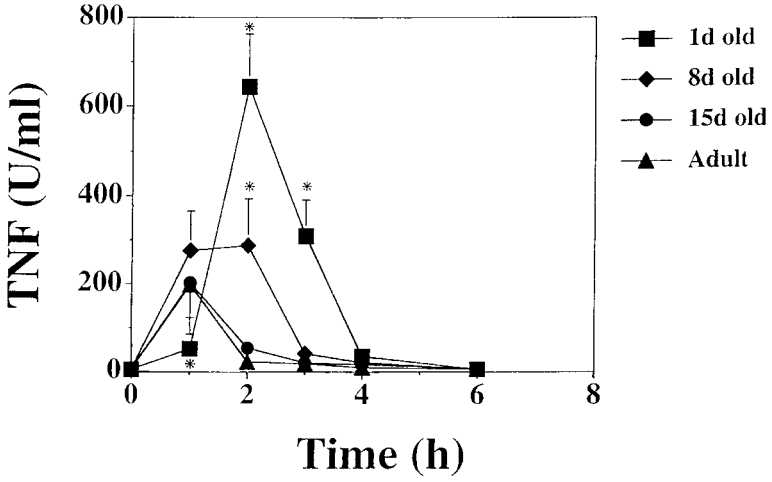

Figure 1. Tumor necrosis factor (TNF) plasma levels in mice of different ages at various times after challenge with lipopolysaccharide. Data are means ± SDs of measurements on 3 different plasma pools, each obtained from 5–10 animals. * Significantly different from adult levels as determined by 1-way analysis of variance and Student-Newman-Keuls test. $P < .05$ was considered significant.

Figure 3 shows that significant elevations of IL-6 levels over baseline were observed after LPS challenge in all age groups. No significant differences were detected between different age groups in the levels of this cytokine, with the exception of lower values in the younger pups at 2 h after LPS. IFN-γ levels (figure 4) were >90% lower ($P < .05$) in 1-, 8-, or 15-day-old animals than in adults. Peak values were observed at 6 h after challenge in all age groups. No significant plasma IL-4 elevations were detected in any of the age groups after LPS injection (not shown).

SEB-induced cytokine production. TNF, IFN-γ, and IL-2 are known to be released in the circulation and to play important roles in the pathophysiology of superantigen-induced shock. Therefore, plasma levels of these cytokines were measured in 1-, 8-, and 15-day-old and adult mice at various times after challenge with 5 mg/kg SEB. This dose was shown to be sufficient, in the presence of D-gal, to kill all of the adult animals but none of the pups (table 3). IFN-γ, IL-2, and TNF elevations (figures 5–7) showed similar kinetics in all age groups. Peak values were always observed at 4 h after challenge. One- and 8-day-old pups produced consistently lower levels of all three cytokines in response to SEB ($P < .05$). In contrast, 15-day-old animals displayed adult-like levels of all of the measured cytokines. Eight-day-old mice displayed intermediate peak levels, which were significantly different from those of either the younger pups or the adults ($P < .05$). Therefore, SEB-induced production of TNF, IFN-γ, and IL-2 closely paralleled lethality in the different age groups. No significant plasma IL-4 elevations were detected in any of the age groups after SEB injection (not shown).

Effects of TNF-α and IFN-γ blockade. TNF or IFN blockade can ameliorate pathophysiologic changes in both SEB- and LPS-induced shock [22, 26, 27]. Since our data indicated age-related differences in cytokine release, we sought to determine if the response to anti-cytokine treatments also differed in adult and neonatal animals. Mice were injected with graded doses

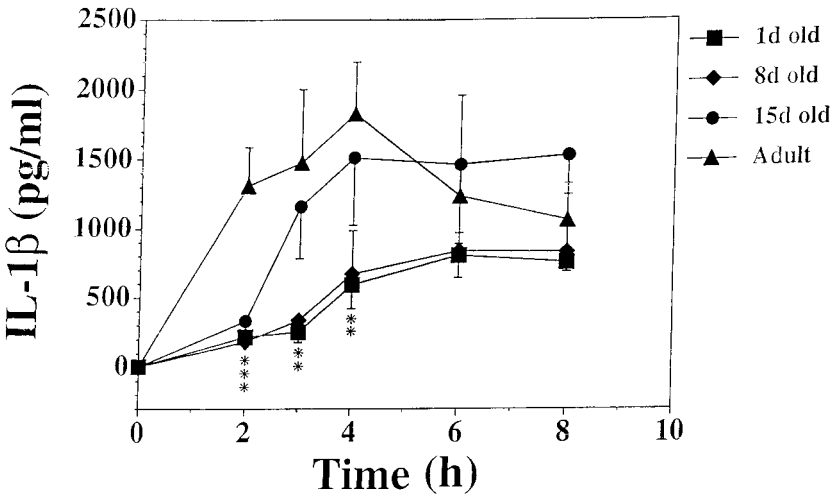

Figure 2. Interleukin-1β (IL-1β) plasma levels in mice of different ages at various times after challenge with lipopolysaccharide. Data are means ± SDs of measurements on 3 different plasma pools, each obtained from 5–10 animals. * Significantly different from adult levels as determined by 1-way analysis of variance and Student-Newman-Keuls test. $P < .05$ was considered significant.

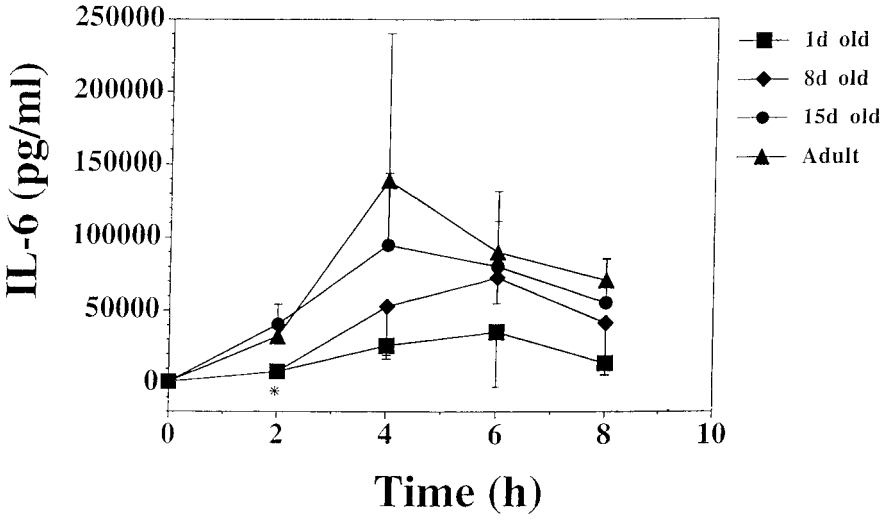

Figure 3. Interleukin-6 (IL-6) plasma levels in mice of different ages at various times after challenge with lipopolysaccharide. Data are means ± SDs of measurements on 3 different plasma pools, each obtained from 5–10 animals. * Significantly different from adult levels as determined by 1-way analysis of variance and Student-Newman-Keuls test. $P < .05$ was considered significant.

of anti–TNF-α rabbit serum 4 h before challenge with LPS or SEB at doses inducing ~50% lethality in controls (table 4). Anti–TNF-α pretreatment protected both neonatal and adult animals against LPS mortality. However 10-times-higher doses were needed to induce complete protection in neonates relative to adults ($P < .05$, table 4).

Anti–TNF-α induced complete protection also against SEB in D-gal-sensitized adults and neonates. However, in contrast with observations in LPS shock, >3-times-higher doses of anti–TNF-α were needed to protect adults relative to neonates. Therefore, observations performed with anti–TNF-α paralleled findings that neonates produce higher levels of TNF in response to LPS and lower levels in response to SEB, relative to adults. These data also indicate a crucial role of TNF-α in neonatal as well adult LPS and SEB shock.

Although plasma IFN-γ levels were very low in neonates inoculated with LPS or SEB, we could not exclude a priori that these low levels or local IFN-γ production affected lethality. Moreover, previous studies had documented IFN-γ production in neonatal mice and rats during infection [28, 29]. Therefore the effects of IFN-γ blockade were compared in adult and neonatal mice (table 5). Anti–IFN-γ protected adult ($P < .05$) but not neonatal mice against LPS toxicity. However anti–IFN-γ did not affect lethality in either adults or neonates challenged with SEB plus D-gal. This lack of IFN-γ blockade should be considered with caution, since D-gal models are characterized by increased sensitivity to TNF-α. However, the lack of effects of anti–IFN-γ in adult, sensitized mice challenged with SEB is in agreement with observations by others [27].

Discussion

The present study documents significant age-related differences in susceptibility to shock-inducing agents. Compared

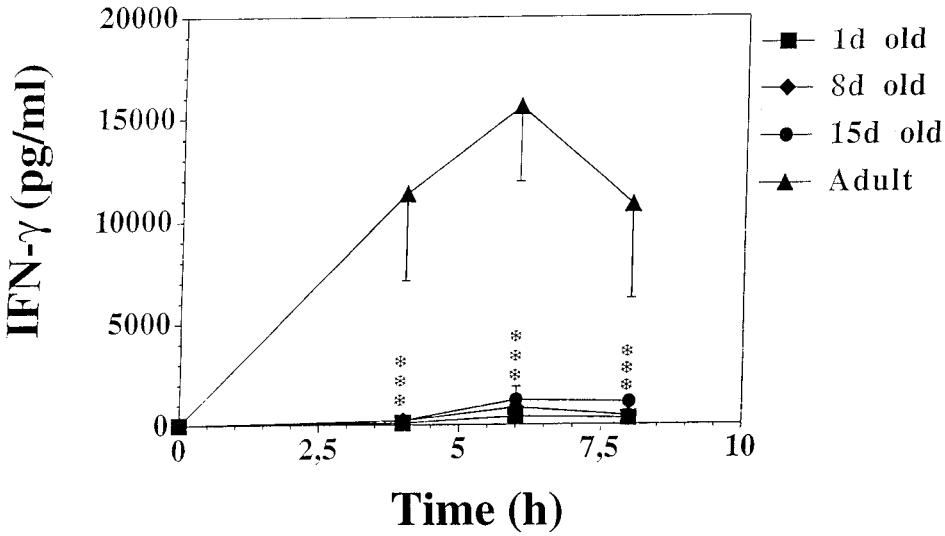

Figure 4. Interferon-γ (IFN-γ) plasma levels in mice of different ages at various times after challenge with lipopolysaccharide. Data are means ± SDs of measurements on 3 different plasma pools, each obtained from 5–10 animals. * Significantly different from adult levels as determined by 1-way analysis of variance and Student-Newman-Keuls test. $P < .05$ was considered significant.

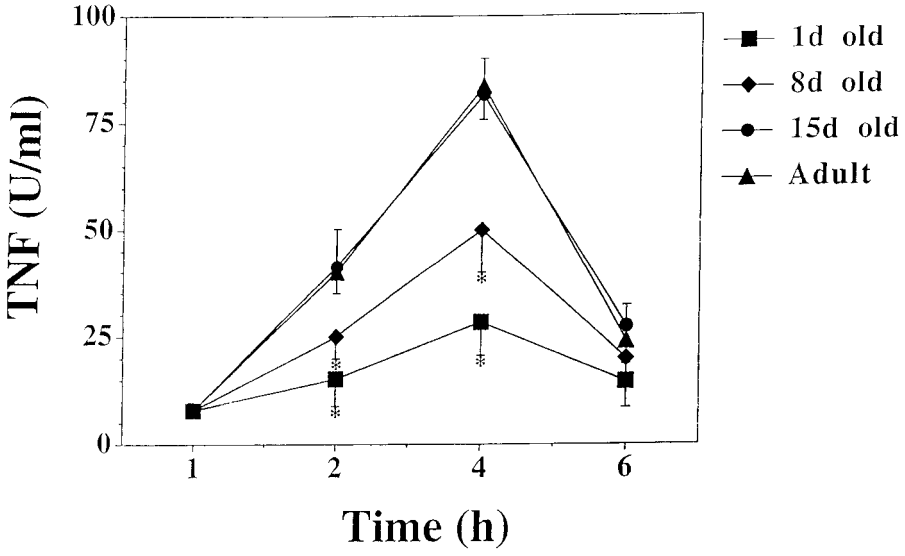

Figure 5. Tumor necrosis factor (TNF) plasma levels in mice of different ages at various times after challenge with staphylococcal enterotoxin B. Data are means ± SDs of measurements on 3 different plasma pools, each obtained from 5–10 animals. * Significantly different from adult levels as determined by 1-way analysis of variance and Student-Newman-Keuls test. $P < .05$ was considered significant.

with adults, neonatal mice were ~10 times more sensitive to the lethal toxicity of LPS but 45 times more resistant to SEB. LPS is considered the main pathogenic factor in infections by gram-negative bacteria, the most frequent cause of neonatal sepsis [30]. Therefore, the increased sensitivity of the newborn to LPS, as well as its immunologic defects in response to infection, may account for the increased incidence of mortality in neonatal sepsis. Conversely, neonates may be less susceptible to shock induced by exotoxin-producing gram-positive bacteria.

Increased susceptibility of neonates to endotoxin was previously documented in rats [31–35] and guinea pigs [36]. The mouse seems a preferable species for this kind of study. The availability of inbred strains with specific defects in the immune system should permit further understanding of the pathophysiology of neonatal shock.

The central finding of this report is that increased neonatal lethality to LPS was associated with higher and more persistent circulating TNF levels. TNF blockade experiments confirmed that TNF-α production is higher in the neonate since 3- to 4-fold-higher doses of anti–TNF-α were needed to protect neonates compared with those required to protect adults. The increased lethality to LPS was not the consequence of increased susceptibility to known mediators of endotoxin toxicity. Both rTNF-α and a combination of rTNF-α and rIFN-γ induced comparable mortality in neonates and adults.

Previous attempts to relate the atypical manifestations of neonatal sepsis to abnormalities in cytokine regulation have yielded contradictory results [9, 37]. The reasons for these discrepancies are not entirely clear but are probably related to differences in composition and purity of the cell populations and culture methods used. Most of these investigations used

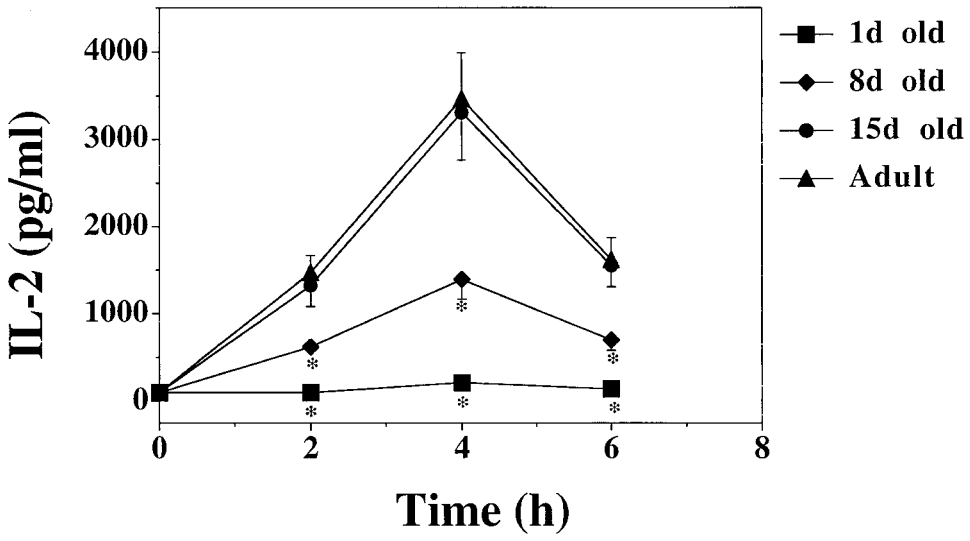

Figure 6. Interleukin-2 (IL-2) plasma levels in mice of different ages at various times after challenge with staphylococcal enterotoxin B. Data are means ± SDs of measurements on 3 different plasma pools, each obtained from 5–10 animals. * Significantly different from adult levels as determined by 1-way analysis of variance and Student-Newman-Keuls test. $P < .05$ was considered significant.

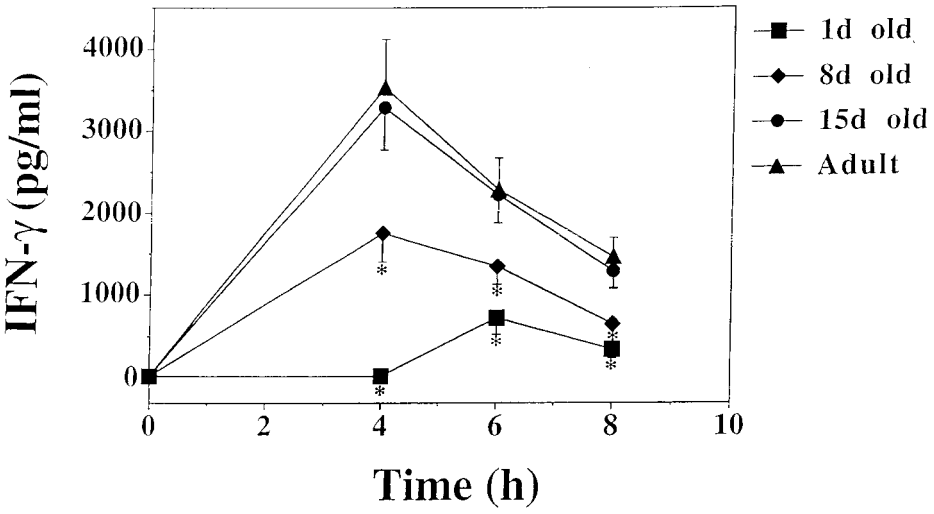

Figure 7. Interferon-γ (IFN-γ) plasma levels in mice of different ages at various times after challenge with staphylococcal enterotoxin B. Data are means $\pm$ SDs of measurements on 3 different plasma pools, each obtained from 5–10 animals. * Significantly different from adult levels as determined by 1-way analysis of variance and Student-Newman-Keuls test. $P < .05$ was considered significant.

human blood cells. Macrophages and several other types of cells present in different tissues, including endothelial cells and fibroblasts, make significant contributions to the overall cytokine production during inflammatory and immune responses. Since cytokine production results from an extensive network of interactions involving many different cell types, in vivo studies also seem important.

Studies are underway to identify cell populations responsible for increased TNF-α production in the neonate in response to LPS. Cell separation studies are complicated by the low yield and atypical physical properties of neonatal cells. Preliminary data, however, indicate that splenic macrophages from neonatal mice produce more TNF-α than those of adults on a per-cell basis (unpublished data).

Neonatal sensitivity to exotoxins has not been previously investigated. In the present study, neonates were highly resistant to SEB challenge, in contrast to their high sensitivity to endotoxin. Unlike LPS, which acts predominantly on macro-

Table 4. Effects of tumor necrosis factor-α (TNF-α) blockade on lipopolysaccharide (LPS)- and staphylococcal enterotoxin B (SEB)–induced lethality in neonatal mice.

Challenge, age	Anti–TNF-α (U/kg $\times$ 10^6)	Lethality (dead/total)
LPS		
1 day	0.0 (NRS)	3/6
	0.2	4/6
	0.6	3/6
	2.0	2/6
	6.0	0/6
Adult	0.0 (NRS)	2/4
	0.2	1/4
	0.6	0/4
	2.0	0/4
	6.0	0/4
SEB + D-galactosamine		
1 day	0.0 (NRS)	3/6
	0.2	1/6
	0.6	0/6
	2.0	0/6
	6.0	0/6
Adult	0.0 (NRS)	3/4
	0.2	2/4
	0.6	1/4
	2.0	0/4
	6.0	0/4

NOTE. Data are cumulative results of 3–5 experiments. Anti–TNF-α was given subcutaneously 4 h before challenge. Animals were challenged with doses of LPS or SEB producing ~50% lethality. Actual doses were: adults, 150 mg/kg LPS and 1 mg/kg SEB + D-galactosamine (350 mg/kg); 1-day-old pups, 15 mg/kg LPS or 50 mg/kg SEB + D-galactosamine (350 mg/kg). NRS, normal rabbit serum.

Table 5. Effect of interferon-γ (IFN-γ) blockade on lipopolysaccharide (LPS)- and staphylococcal enterotoxin B (SEB)–induced lethality in neonatal mice.

Challenge, age	Anti–IFN-γ (U/kg $\times$ 10^6)	Lethality (dead/total)
LPS		
1 day	0.0 (NHIgG)	4/6
	15.0	4/6
Adult	0.0 (NHIgG)	5/6
	15.0	0/6
SEB + D-galactosamine		
1 day	0.0 (NHIgG)	3/6
	15.0	4/6
Adult	0.0 (NHIgG)	4/6
	15.0	3/6

NOTE. Data are cumulative results of 3–5 experiments. Anti–IFN-γ was given subcutaneously 4 h before challenge. Animals were challenged with doses of LPS or SEB producing ~50% lethality. Actual doses were: adults, 150 mg/kg LPS and 1 mg/kg SEB + D-galactosamine (350 mg/kg); 1-day-old pups, 15 mg/kg LPS or 50 mg/kg SEB + D-galactosamine (350 mg/kg). NHIgG, normal hamster IgG.

phages [21], SEB and other superantigenic exotoxins induce shock by stimulating mainly T cells. Reconstitution studies in nude mice show that they can be sensitized to SEB by the transfer of T cells [22].

Increased resistance to SEB in the present study correlated with decreased plasma levels of TNF, IFN-γ, and IL-2. The decreased neonatal production of these cytokines after SEB may be a consequence of reduced Th1 responses during early life. T cells from 4-day-old mice were previously reported to produce minimal IL-2 and IFN-γ but high IL-4 levels in response to primary stimulation in vitro with anti-CD3 antibody [8]. Down-regulation of IL-2 in the neonatal period was the result of a combination of intrinsic unresponsiveness to CD3-mediated stimulation and high production of IL-4. Our inability to detect significant elevations of circulating IL-4 in response to either LPS or SEB does not rule out that local T cell responses may involve higher tissue IL-4 production in the neonatal period.

It was suggested that IFN-γ is the ultimate mediator of lethality in a number of shock models [26]. Previous studies have also indicated that IFN-γ is produced in neonatal rats and mice during infection and that this response is TNF-α–dependent [28, 29]. Therefore, it was of interest to assess the role of IFN-γ in neonatal shock. It was found here that anti–IFN-γ was effective in protecting adult but not neonatal mice against LPS challenge. These data indicate that IFN-γ is not an obligatory mediator of disease processes initiated by endotoxin or TNF-α and that neonatal shock models are relatively IFN-γ–independent.

Recent data in a live gram-positive bacteria model also indicate that endogenous IFN-γ does not play a major pathophysiologic role in neonatal sepsis [28]. Not only was IFN-γ blockade ineffective in the latter model, but administration of rIFN-γ improved survival and bacterial clearance.

Neonates are often unable to mount a febrile response, even in the course of severe infections [2]. IL-1, TNF-α, and IL-6 are considered the main mediators of fever. In the present study, maximal levels of IL-1β were significantly decreased in 1- to 8-day-old pups compared with levels in adults in response to LPS. IL-1 production reached adult-like values at 2 weeks after birth. It is possible that a relative inability of neonates to produce IL-1β is at least partially responsible for their low febrile responses. Interestingly, high levels of TNF-α and IL-6 were detected in human neonatal sepsis, whereas IL-1β appeared to be present in small amounts only [38]. Although no differences were detected between the IL-1β secretion of peripheral blood monocytes from preterm and term neonates and that of monocytes from adults [11], IL-1β production was decreased in infants with infectious complications postpartum [39].

In conclusion, our data document significant age-related changes in susceptibility to shock-inducing gram-negative and gram-positive agents. Neonatal mice were exquisitely sensitive to LPS but highly resistant to SEB. This could reflect differences in the cell types that are preferentially stimulated by these agents. In both neonates and adults, however, LPS and SEB lethality correlated with elevated plasma TNF levels, which was confirmed by TNF blockade experiments. In contrast, IFN-γ may not be an obligatory mediator of lethality in neonatal shock. Since neonatal sepsis has a high incidence of serious complications despite aggressive treatment, these observations may be useful in developing alternative therapeutic strategies.

References

1. Saez-Llorens X, Ramilo O, Mustafa MM, Mertsola J, McCracken GH. Molecular pathophysiology of bacterial meningitis: current concepts and therapeutic implications. J Pediatr **1990**;116:671–84.
2. Felgin RD, Adcock LM, Miller DJ. Postnatal bacterial infections. In: Fanaroff AA, Martin RJ, eds. Neonatal-perinatal medicine: diseases of the fetus and infant. 5th ed. St. Louis: Mosby-Year Book, **1992**:619–61.
3. Redding JL, Starzecki B, Spink BWW. Comparative hemodynamic and humoral responses of puppies and adult dogs to endotoxin. Am J Physiol **1966**;210:540–5.
4. Jia-Xian L, James RO, Chong-Yuan L, Kimberly DG, Philips JB III. Age-relative differences in responses to endotoxin infusion in unanesthetized piglets. Circ Shock **1993**;41:40–7.
5. Bryson YJ, Winter HS, Gard SE, Fischer TJ, Stehm ER. Deficiency of immune interferon production by leukocytes of normal newborns. Cell Immunol **1980**;55:191–200.
6. Seki H, Taga K, Masuda N. Phenotypic and functional characterization of active suppressor cells against IFN-gamma production in PHA-stimulated cord blood lymphocytes. J Immunol **1986**;137:3158–67.
7. Wilson CB, Westall J, Johnston L, Lewis DB, Dower SK, Alpert AR. Decreased production of interferon gamma by human neonatal cells: intrinsic and regulatory deficiencies. J Clin Invest **1986**;77:860–7.
8. Adkins B, Ghanei A, Hamilton K. Developmental regulation of IL-4, IL-2, and IFN-γ production by murine peripheral T lymphocytes. J Immunol **1993**;151:6617–26.
9. Pillay V, Savage N, Laburn H. Circulating cytokine concentrations and cytokine production by monocytes from newborn babies and adults. Pflugers Arch **1994**;428:197–201.
10. English BK, Burchett SK, English JD, Ammann AJ, Wara DW, Wilson CB. Production of lymphotoxin and tumor necrosis factor by human neonatal mononuclear cells. Pediatr Res **1988**;24:717–21.
11. Weatherstone KB, Rich EA. Tumor necrosis factor/cachectin and interleukin-1 secretion by cord blood monocytes from premature and term neonates. Pediatr Res **1989**;25:342–6.
12. Williams PA, Bohnsack JF, Augustine NK, Drummond WK, Rubens CE, Hill HR. Production of tumor necrosis factor by human cells in vitro and in vivo, induced by group B streptococci. J Pediatr **1993**;123:292–7.
13. Bessler H, Sirota L, Dulitzky F, Djaldett M. Production of interleukin 1 by mononuclear cells of newborns and their mothers. Clin Exp Immunol **1987**;68:655–61.
14. Burchett SK, Weaver WM, Westall JA, Larsen A, Kronheim S, Wilson CB. Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. J Immunol **1988**;140:3473–81.
15. Dinarello CA, Shipbarber M, Kent EFJ, Wolff SM. Production of leukocytic pyrogen from phagocytes of neonates. J Infect Dis **1981**;144:337–45.
16. Glover DM, Brownstein D, Burchett S, Larsen A, Wilson CB. Expression of HLA class II antigens and secretion of interleukin-1 by monocytes and macrophages from adults and neonates. Immunology **1987**;61:195–201.

17. Wilmott RW, Harris MC, Haines KM, Douglas SD. Interleukin-1 activity from human cord blood monocytes. Diagn Clin Immunol **1987**;5: 201–7.
18. Schibler KR, Liechty KW, White WL, Rothstein G, Christensen RD. Defective production of interleukin-6 by monocytes: a possible mechanism underlying several host defence deficiencies of neonates. Pediatr Res **1992**;31:18–21.
19. Yachie A, Takano N, Ohta K, et al. Defective production of interleukin-6 in very small premature infants in response to bacterial pathogens. Infect Immun **1992**;60:749–53.
20. Yachie A, Takano N, Yokoi T, et al. The capability of neonatal leukocytes to produce IL-6 on stimulation assessed by whole blood culture. Pediatr Res **1990**;27:227–33.
21. Freudenberger M, Keppler D, Galanos C. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect Immun **1986**;51:891–5.
22. Miethke T, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. J Exp Med **1992**;175:91–8.
23. Espevik T, Nissen-Meyer J. A highly sensitive cell line WEHI 164 clone 13 for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods **1986**;95:99–105.
24. Mancuso G, Tomasello F, Migliardo M, et al. Beneficial effects of interleukin-6 in neonatal mouse models of group B streptococcal disease. Infect Immun **1994**;62:4997–5002.
25. Schlech WF. Toxic shock syndrome. In: Hardaway RM, ed. Shock: the reversible stage of dying. Littleton, MA: PSG Publishing, **1988**:303.
26. Doherty GM, Lange JR, Langstein HN, Alexander R, Buresh CM, Norton JA. Evidence for IFN-γ as a mediator of the lethality of endotoxin and tumor necrosis factor-α. J Immunol **1992**;149:1666–70.
27. Matthys P, Mitera T, Heremans H, Van Damme J, Billiau A. Anti-gamma interferon and anti–interleukin-6 antibodies affect staphylococcal enterotoxin B–induced weight loss, hypoglycemia, and cytokine release in D-galactosamine–sensitized and unsensitized mice. Infect Immun **1995**;63:1158–64.
28. Cusumano V, Mancuso G, Genovese F, et al. Role of interferon-γ in a neonatal mouse model of group B streptococcal disease. Infect Immun **1996**;64:2941–4.
29. Teti G, Mancuso G, Tomasello F. Cytokine appearance and effects of anti–tumor necrosis factor alpha antibodies in a neonatal rat model of group B streptococcal infection. Infect Immun **1993**;61: 227–33.
30. Jarvis WR. Epidemiology of nosocomial infections in pediatric patients. J Pediatr **1987**;6:344–51.
31. Cochran JB, Chen H, La Via M, Cusumano V, Teti G, Cook JA. Age-related mortality and adherent splenic cell mediator production to endotoxin in the rat. Shock **1995**;4:450–4.
32. Fitzgerald M, Zeller WP, Goto M, Anderson CL, Hurley RM. Concurrent clinical and metabolic derangements in the newborn rat: a late phase sepsis model. Ann Clin Lab Sci **1988**;18:229–34.
33. Porter PJ, Spievack AR, Kass EH. The effect of neonatal thymectomy on susceptibility to bacterial endotoxin. J Lab Clin Med **1966**;68:455–62.
34. Zeller WP, Goto M, Witek JL, Hurley RM. Mortality, temporal substrate and insulin responses to endotoxic shock in zero, ten, and twenty-eight day old rats. Surg Gynecol Obstet **1991**;173:375–83.
35. Mancuso G, Tomasello F, von Hunolstein C, Orefici G, Teti G. Induction of tumor necrosis factor alpha by the group- and type-specific polysaccharides from type III group B streptococci. Infect Immun **1994**;62: 2748–53.
36. Uhr JW. The effect of bacterial endotoxin on the newborn guinea pig. J Exp Med **1962**;115:685–94.
37. Schibler KR, Liechty KW, White WL, Rothstein G, Christensen RD. Defective production of interleukin 6 by monocytes: a possible mechanism underlying several host defence deficiencies of neonates. Pediatr Res **1994**;31:18–21
38. De Bont ESJM, Martens A, van Raan J, et al. Tumor necrosis factor-α, interleukin 1β, and interleukin-6 plasma levels in neonatal sepsis. Pediatr Res **1993**;33:380–3.
39. Miller LC, Sana I, Lo Preste G, Schaller JG, Dinarello CA. Neonatal interleukin-1β, interleukin 6, and tumor necrosis factor: cord blood levels and cellular production. J Pediatr **1990**;117:961–7.