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Neonatal Host Defense Mechanisms against *Listeria monocytogenes* Infection: The Role of Lipopolysaccharides and Interferons

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ABSTRACT. The human newborn infant is susceptible to lethal infection caused by a number of bacterial species including *Listeria monocytogenes*, a gram-positive rod which is pathogenic by virtue of its ability to survive intracellularly. In adult animals interferon (IFN)-α/β and IFN-γ or agents that induce or augment IFN production confer protection against lethal *L. monocytogenes* infection. Regulation and production of IFN is poorly understood during the neonatal period. We therefore evaluated the role of IFN-α/β and IFN-γ, IFN-inducers (polyinosinic:polycytidylic acid, amino-bromo-phenyl-pyrimidinone, amino-iodophenyl pyrimidinone) and lipopolysaccharide in modifying neonatal *L. monocytogenes* infection. Pretreatment of juvenile rats with polyinosinic:polycytidylic acid or lipopolysaccharide protected them against a lethal challenge with *L. monocytogenes*. Among newborn rats, polyinosinic: polycytidylic acid, amino-iodo-phenyl pyrimidinone and amino-bromophenyl-pyrimidinone gave significant protection, however, lipopolysaccharide did not influence survival. The role of IFN was further examined. Pretreatment of 3-d-old rats with purified IFN-α/β, native rat IFN-γ or rDNA rat IFN-γ protected them against the lethality of subsequent *L. monocytogenes* injection. At 3 d after bacterial challenge, bacterial content in the spleens of 3-d-old rats pretreated with rIFN-γ were significantly decreased compared to controls: IFN-α/β-pretreated animals had less of a decrease, which become significant only 5 d after challenge. Our experiments indicate a role for IFN in neonatal host defense against *L. monocytogenes* infection. (*Pediatr Res* 25:311–315, 1989)

Abbreviations

IFN, interferon
polyI/C, polyinosinic:polycytidylic acid
ABPP, amino-bromo-phenyl-pyrimidinone
AIPP, amino-iodo-phenyl-pyrimidinone
LPS, lipopolysaccharide
CFU, colony forming units of bacteria
ip, intraperitoneal
sc, subcutaneous
TNF, tumor necrosis factor

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Listeria monocytogenes, the causative agent of listeriosis, is a short gram-positive motile rod believed to be pathogenic by virtue of its ability to survive intracellularly. For adult immunocompetent animals, the process leading to development of resistance has been partially elucidated. Within 3–5 d after *L. monocytogenes* infection has been introduced, there is a rapid proliferation of reactive T-lymphocytes (1–4). Resistance to Listeria infection appears to be mediated by a T-lymphocyte-produced lymphokine which induces increased macrophage and natural killer cell activity (5–7), which then become Listericidal (8, 9). Interferons, peptide molecules capable of affecting a number of cellular functions, are among the major lymphokines activating these cells.

In mature animals *L. monocytogenes* infection induces circulating IFN-α/β soon after the organism is injected (10–12). Peak immunity to *L. monocytogenes*, which is evident on or about the 6th d of infection, coincides with the production of IFN-γ by the spleen (4, 13, 14). Recently, Havell (13) has also shown that interferon production during *L. monocytogenes* infection is greatly augmented by LPS, and he suggested this as an explanation for the increased resistance to *L. monocytogenes* infection that is observed for animals pretreated with LPS (15).

It has recently been shown that human neonatal lymphocytes produce little IFN-γ in response to mitogens (16, 17). Decreased production of IFN-γ by neonatal cells appears to be due both to intrinsic capacity to produce IFN- and to regulatory mechanisms (16, 17). In addition, neonatal monocytes and monocyte-derived macrophages, important target cells for IFN-γ, are less active than adult cells (18, 19).

The human neonate is unusually susceptible to infection with *L. monocytogenes*. The reason for this is not clear. Because of the role of IFN and LPS in modifying adult *L. monocytogenes* infection, we investigated the effects of LPS, IFN, and IFN-inducers in the development of resistance to *L. monocytogenes* using a newborn rat model of neonatal infection (20, 21). Our studies support an important role for IFN-α/β and IFN-γ in newborn *L. monocytogenes* infection and also demonstrate a deficiency for newborn animals to respond to LPS.

MATERIALS AND METHODS

Reagents. LPS was obtained from Difco Laboratories (Detroit, MI). PolyI/C was obtained from P.L. Biochemicals Inc. (Rochester, NY). ABPP and AIPP were kindly provided by Upjohn Company (Kalamazoo, MI). Rat IFN-α/β was purchased from Lee Biologics (San Diego, CA). Rat rIFN-γ is the product of transfection with a plasmid carrying the chromosomal rat IFN-γ gene as described elsewhere (22). The rIFN-γ was purified to >98% purity by immunoaffinity chromatography using MAb bound to sepharose. Native rat IFN-γ was produced as described elsewhere by treating rat lymphocytes from lymph nodes and

spleen with neuraminidase and galactose oxidase (23). The cells were then incubated for 48 h, and the cultured supernatant was purified by hydroxylappatite chromatography to a sp act of >500 000 U/mg.

LPS (O55:B5) was mixed with PBS to a concentration of 1 mg/mL, and this stock was stored in aliquots at −20°C. Stock aliquots were diluted 1/100–1/1000 for animal inoculation. PolyI/C was solubilized in PBS by heating a 2 mg/mL mixture to 50°C for 15–20 min or until the solution clarified. Stock solution was diluted in PBS for injection into animals. ABPP and AIPP were prepared using sterile preservative-free 0.5% methyl cellulose (2500 μg/ml). The material was sonicated for 1–2 min to distribute the drug throughout the methyl cellulose. Fresh dilutions in methyl cellulose were prepared for each treatment. Rat IFN-α/β was stored at 4°C in aliquots, then diluted in sterile PBS for injection. Rat IFN-γ was stored at −70°C in aliquots, then diluted in PBS with 0.5% BSA (PBS-BSA) for injection into animals. Controls used for each preparation were injected with identical vehicles which lacked the active agent.

Bacteria. A strain of *L. monocytogenes* type 4b designated 15U in previous publications (20, 21) and isolated originally from the blood of an infant with neonatal listeriosis was used in these studies. Aliquots of a stock culture were stored in 20% glycerol and frozen at −20°C until required for animal studies. A portion of the frozen aliquot was inoculated into brain heart infusion broth (Difco) and incubated at 37°C overnight. The organism was then sedimented (3000 × *g* for 10 min) and washed once in PBS. The number of bacteria was estimated spectrophotometrically and verified using pour plate technique.

In vivo studies. Timed pregnant outbred Sprague-Dawley rats (Canadian Hybrid Farms, Hall's Harbour, Nova Scotia, Canada) were obtained at the 13th d of gestation and fed rat food *ad lib* and housed in a controlled environment (16 h light/d) until parturition (usually 21st–22nd d of gestation). Pups that were 3-d-old or 30-d-old were randomly allocated to receive one of the treatment regimes. To minimize inter-litter variability, no more than three animals in each litter received the same treatment regime. Rats that were 3-d-old were injected with 10^6 CFU of *L. monocytogenes* ip or with 10^8 CFU subcutaneously, an inoculum that killed approximately 90% of untreated animals. Most deaths occurred 2–5 d after bacterial challenge. Animals that were 30-d-old were injected with 10^9 CFU of *L. monocytogenes* ip, an inoculum that caused 50% mortality. A larger inoculum in 30-d-old rats resulted in overwhelming bacterial infection and deaths within 48 h of injection. The dose of 10^9 was chosen to simulate the dynamics of infection better in 3-d-old rats in which deaths occur on d 3 or 4 after challenge. In some experiments, blood cultures were obtained by cardiac puncture at the time the animal was killed by carbon dioxide inhalation. Quantitation of blood bacterial content was done using pour plates of serial dilutions of blood. Results were expressed as CFU/mL of blood. In some animals, the spleen was dissected free, weighed, and homogenized in PBS. Pour plates were prepared using serial dilutions from the splenic homogenates and bacterial content expressed as CFU/100 mg of spleen. Pour plates of tissue used brain heart infusion agar (Difco) containing 3 μg/mL moxolactam (Lilly Pharmaceutical, Toronto, Canada) to suppress any contaminating bacteria. This level of antibiotic did not affect *L. monocytogenes* viability.

Statistical methods. Analysis of groups was done using a 2-sided paired *t* test for comparison of bacteria in tissue. A chi-square test or the Fisher exact test were used to compare survival of various groups. Significance was accepted for $p < 0.05$.

RESULTS

As shown in Figure 1, pretreatment of juvenile rats with polyI/C (50 μg/kg body wt) given ip 2 and 1 d before and on the d of bacterial challenge or LPS (20 μg/kg body wt) given sc 1 d before challenge significantly improved the animals' survival calculated

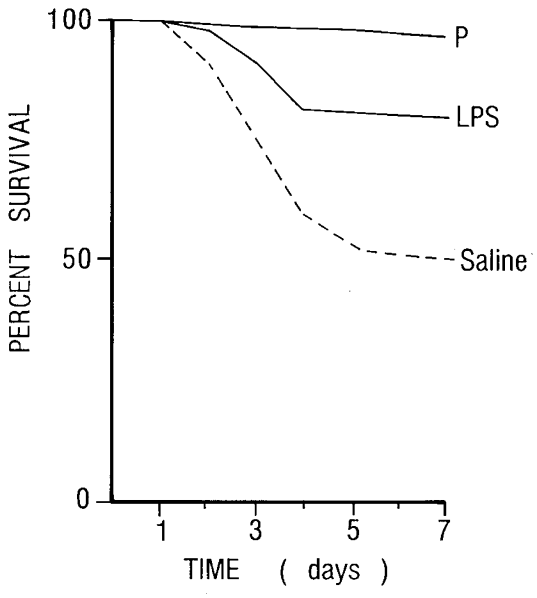

Fig. 1. Survival of 30-d-old animals after *L. monocytogenes* injection. The percentage of survival of 30-d-old animals for 7 d after ip injection of 1×10^9 CFU *L. monocytogenes* is shown. Animals had been pretreated ip with polyI/C (P, 50 μg/kg body wt) or sc with LPS (20 μg/kg body wt); $p < 0.001$ and $p < 0.005$ for polyI/C and LPS *versus* saline, respectively.

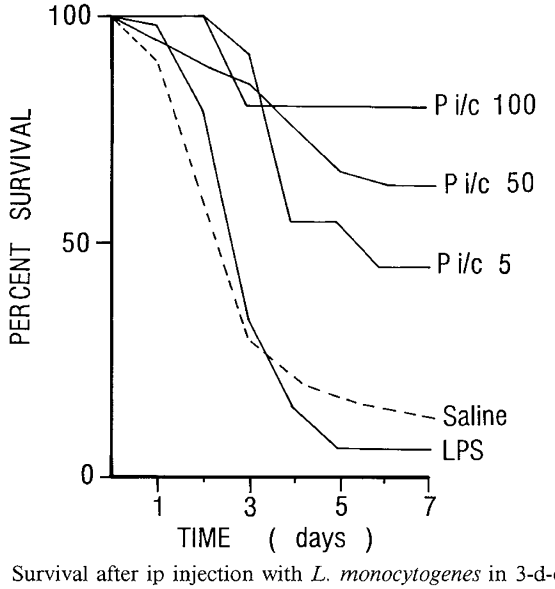

Fig. 2. Survival after ip injection with *L. monocytogenes* in 3-d-old (newborn) rats. Newborn rats that had been pretreated ip with polyI/C (*Pi/c 100, 50* or *5* μg/kg of body wt), sc with LPS (L, 20 μg/kg body wt), or PBS (*saline*) were injected ip on day 3 of life with 10^6 CFU of *L. monocytogenes.* The percentage survival was calculated for 7 d after bacterial challenge. $p < 0.001$ for polyI/C 50 and 100 μg/kg *versus* saline and $p < 0.05$ for polyI/C 5 μg *versus* saline.

7 d after injection ($p < 0.001$ and $p < 0.005$, respectively). Among newborn animals (Fig. 2), the effects of polyI/C (50 μg/kg body wt) were similar to those seen with juveniles ($p < 0.001$ *versus* controls). A dose-related response to polyI/C was shown for doses of 5–100 μg/kg animal wt. In addition, a protective effect of polyI/C was observed when bacteria and polyI/C were administered at different sites (Table 1). However, unlike the juvenile animals, 3-d-old pups had no protective effect from LPS (Fig. 1). No benefit was observed for higher or lower doses of

Table 1. *Effect of various agents on survival of intraperitoneally L. monocytogenes-infected newborn rats*

Agent*	Route of treatment	Treatment group: No.: surviving/injected (% survival)	Controls: No.: surviving/injected (% survival)
Poly I/C			
50 μg/kg	ip	35/55 (64)†	12/93 (13)
50 μg/kg	sc	7/33 (21)	7/56 (12)
500 μg/kg	sc	9/10 (90)†	7/56 (12)
ABPP			
2.5 mg/kg	ip	4/20 (20)	1/17 (6)
1.25 mg/kg	po	6/10 (60)‡	2/14 (14)
AIPP			
2.5 mg/kg	ip	10/19 (53)†	1/17 (6)
1.25 mg/kg	po	2/10 (20)	2/14 (14)
LPS			
20 μg/kg	sc	3/52 (6)	7/56 (12)

* Except for LPS, all agents were injected ip on d 2 and 1 before and the d of bacterial challenge. For LPS, animals were injected sc 1 d before bacterial challenge.

† $p < 0.01$ *versus* control, chi-square test or Fisher exact test if groups less than 20.

‡ $p < 0.05$ *versus* control, Fisher exact test.

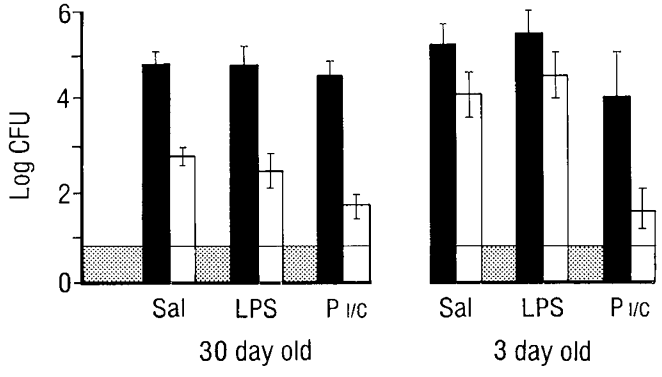

Fig. 3. Splenic and blood *L. monocytogenes* concentration. Mean log CFU ± SEM in spleen (per 100 mg tissue, *solid bars*) or in blood (per ml blood, *open bars*) for 30-d-old and 3-d-old rats pretreated with PBS (*Sal*), LPS, or polyI/C (*PI/C*). Cultures were obtained 3 d after bacterial challenge. *Stippled area* shows the limit of sensitivity for the assay.

LPS or of bacteria (Bortulossi R, Issekutz T, Burbridge S, Schellekens H, manuscript in preparation).

The results of quantitative cultures of splenic tissue and blood obtained 3 d after bacterial challenge from juvenile and newborn animals are shown in Figure 3. The geometric mean bacterial content in spleens was similar for 30-d-old and 3-d-old animals. In both age groups, animals pretreated with polyI/C had a lower content of bacteria. In contrast to this, a marked difference in the bacterial content of blood was found for the two age groups. The 3-d-old animals treated with PBS or LPS had higher bacterial content in blood. Animals pretreated with polyI/C had a lower content of bacteria in blood, in a range similar to that found in older animals.

Other IFN-inducers (ABPP and AIPP) suspended in methyl cellulose (2500 μg/ml), were assessed using, as control, animals treated with the same carrier without the active agent. Animals that were 3-d-old were injected ip with 0.1 mL (250 μg) or were fed approximately 0.05 mL (125 μg) of the compounds three times in total, 2 and 1 d before and on the d of bacterial challenge. As shown in Table 1, the survival of animals fed ABPP was 60% compared to 14% for controls ($p < 0.05$); survival for animals fed AIPP was 20% ($p > 0.05$ *versus* control animals). When animals were given the drugs ip, AIPP was more effective in preventing death than ABPP (53% *versus* 20% survival, Table 1).

In subsequent experiments, purified IFN-α/β and native rat IFN-γ were administered ip using the same schedule for inoculation as with polyI/C. Both IFN-γ and IFN-α/β were found to be protective in newborn rats in a dose-related fashion. IFN (10^5 U/kg) of either type were protective (Table 2).

The dynamics of IFN-induced protection of *L. monocytogenes*-infected newborn pups were studied further by killing animals and determining the bacterial burden in spleen 1, 3, 5, or 7 d after they had been inoculated with an LD_{25} dose of bacteria. Rat rIFN-γ was used in these studies in place of native rat IFN-γ. The bacterial content in the spleens at 1, 3, and 5 d was significantly decreased for animals receiving rIFN-γ; animals receiving IFN-α/β were not significantly different from controls until d 5. In addition, the number of injections appears to correlate with the clearance of bacteria from spleen. The geometric mean CFU in spleens increased as the number of IFN injections decreased (see Table 3).

DISCUSSION

At least three stages of host response to Listeria occur in normal adult animals (24). Within hours after intravenous injection of organisms, the liver and spleen capture and destroy 50% to 90%

Table 2. *Effect of IFN on survival of L. monocytogenes-infected newborn rats*

IFN type*	U/kg	Treatment route*	Challenge route†	Treatment groups: No.: surviving/injected (% survival)	Controls: No.: surviving/injected (% survival)
α/β	10^5	ip	ip	10/10 (100)‡	3/18 (17)
	10^3	ip	ip	10/19 (53)§	3/18 (17)
	10^5	ip	sc	1/7 (14)‖	0/6 (0)
γ	10^5	ip	ip	10/10 (100)‡	3/18 (17)
	10^3	ip	ip	2/10 (20)	3/18 (17)
	10^5	ip	sc	6/7 (86)¶	0/6 (0)

* IFN-α/β or IFN-γ were injected by the route indicated on d 2 and 1 before and the d of bacterial challenge.

† 10^6 CFU *L. monocytogenes* were injected either ip or 10^8 CFU were injected sc.

‡ $p < 0.01$ *versus* control (Fisher exact test).

§ $p < 0.05$ *versus* control (Fisher exact test).

‖ $p < 0.05$ *versus* IFN-γ (Fisher exact test).

¶ $p < 0.05$ *versus* IFN-α/β and *versus* controls (Fisher exact test).

Table 3. *Effect of number of IFN doses on bacterial content in spleen*

No. treatment doses*	CFU/100 mg spleen	
	IFN-γ†	IFN-α/β‡
3	1.6×10^2	5.6×10^4
2	10.9×10^2	9.3×10^4
1	32.4×10^2	17.8×10^4

* Animals were injected ip 3 times (on d 2 and 1 before and on the d of bacterial challenge), 2 times (and 1 before and on the d of bacterial challenge), or 1 time (on the d of bacterial challenge).

† 10^3 U IFN-γ was injected ip on the d indicated, and the geometric mean of bacteria in spleen was counted for four animals in each group that were killed 3 d after bacterial challenge.

‡ 10^3 U IFN-α/β was injected ip on the d indicated, and the geometric mean of bacteria in spleen was counted for four animals in each group that were killed 3 d after bacterial challenge.

of the inoculum. The surviving bacteria multiply in macrophage cells of the liver and spleen for 2 or 3 more d. In the normal mature host, specific T-cell immunity (activation) develops after this (1–3, 6, 15, 25). Once activated, immune T-cells secrete IFN-γ and other lymphokines having immunomodulating effects (4, 7, 14, 23, 26).

The role of IFN-α/β and IFN-γ has been demonstrated for adult mice (13, 27, 28). Mice infected intravenously with an immunizing dose of *L. monocytogenes* produced circulating IFN-α/β during the inductive phase of immune response. In addition, mice acquire a 50-fold greater capacity to produce IFN-α/β after intravenous injection of endotoxin. Finally, peritoneal exudate cells and splenic cells of mice infected with *L. monocytogenes* show increased production of IFN-γ after antigen or mitogen stimulation. The increased production of IFN-γ correlates with the clearance of Listeria from the peritoneal cavity and spleen. Neutralizing MAb to IFN-γ inhibits the generation of activated macrophages *in vitro* (14). *In vivo*, MAb also abrogates clearance of bacteria from the spleen and peritoneal cavity (27).

Pretreatment of adult mice with LPS has also been demonstrated to enhance protection against death due to Listeria (15). LPS has also been shown to increase the accumulation of activated mononuclear phagocytic cells in the liver (29) and the production of TNF from monocytes (30). Because neonatal cells have intrinsic and regulatory deficiencies in IFN-γ production (17, 26) and because LPS may have a synergistic modulating effect on host immunity (13, 29, 30), we investigated these two factors in newborn immunity to *L. monocytogenes*.

Initially we demonstrated that juvenile (30-d-old) rats were protected from *L. monocytogenes* infection when pretreated with LPS or the IFN-inducer, polyI/C. Such animals showed significant improvement in survival and a decreased bacterial load in splenic tissue. Bacterial content in blood was relatively low in both untreated and treated juvenile animals, approximately 1/1000 of spleen (CFU/100 mg spleen *versus* CFU/mL blood). When 3-d-old rat pups were similarly pretreated, a significant protective effect was seen with polyI/C only. In contrast to older animals, LPS had no protective effect (Fig. 1). In addition, both untreated and LPS-treated animals had similar bacterial content in blood, approximately 10-fold higher than in juvenile animals (Fig. 3). In this respect, 3-d-old polyI/C treated animals were similar to untreated juvenile animals (Fig. 3).

Other IFN inducing agents (AIPP and ABPP) were protective as well. With AIPP, protection was only demonstrated when the drug was administered at the same site as bacteria (ip), while ABPP protection was best when it was given by mouth. Oral administration of ABPP induces high levels of circulating IFN in several animal species (31). By contrast, AIPP is one of the most active antiviral agents when given ip but lacks activity when given orally (32). Overall, the results of experiments using AIPP

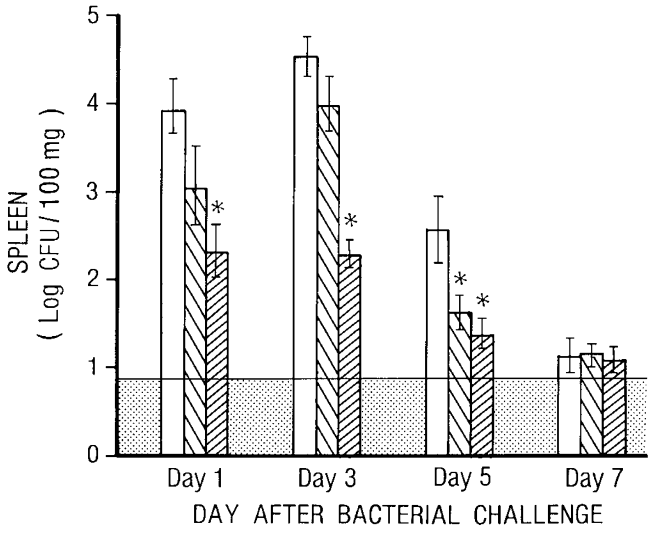

Fig. 4. Effect of interferon pretreatment on splenic concentration of *L. monocytogenes*. Mean log CFU ± SEM in spleen (per 100 mg tissue) for newborn rats pretreated with PBS (□), IFN-α/β (▨) or IFN-γ (▨) before challenge with bacteria. Animals were killed at different times after ip bacterial challenge. Stippled area shows the limit of sensitivity for the assay. *$p < 0.05$ treated *versus* controls.

and ABPP support a role for IFN in the protection that was seen in newborn rats. However, Anthony *et al.* (33) have suggested that the effect of polyI/C, ABPP, and AIPP may act by non-IFN mechanisms in inducing Listeria protection. In addition, circulating IFN is found only in low concentration after these agents are administered to rats (34). To determine the importance of IFN-α/β and IFN-γ more precisely, studies were repeated using purified rat IFN-α/β, IFN-γ, and rat rIFN-γ. A dose-related protective effect was shown (Table 2). The dose required to give complete protection in rats was similar for IFN-α/β and -γ (10^5 U/kg); however, only IFN-γ was effective if bacteria were given at a different site. The maximum effects of IFN were demonstrated when it was administered on 3 consecutive d before bacterial challenge (Table 3). With IFN-γ, significant benefit was present within 1 d of bacterial challenge; benefit was not seen for IFN-α/β until 5 d (Fig. 4). This suggests to us that the effect of IFN-α/β may be dependent on other factor(s) that are induced or augmented by it.

Our experiments therefore further support the role for IFN-α/β and -γ in the host immune response to *L. monocytogenes* infection. Newborn rats respond to IFN-inducers, polyI/C, and ABPP, by increasing their resistance to *L. monocytogenes* infection. The dynamics of bacterial infection in newborn rats after pretreatment with polyI/C mimics that seen among untreated juvenile animals. Although, as proposed by Anthony *et al.* (33), polyI/C and ABPP may have anti-Listeria activity unrelated to IFN production, our study suggests that IFN does play a role in our animal model. That this effect was related to IFN was supported by experiments using purified IFN-α/β or rat rIFN-γ. Newborn rats therefore appear to be protected by IFN against overwhelming *L. monocytogenes* infection. The role of LPS in neonatal protection against *L. monocytogenes* infection is less clear. Pretreatment of juvenile animals with LPS led to significant protection against subsequent bacterial challenge. Similar treatment of 3-d-old animals with varying doses of LPS had no effect. Recently it has been shown that newborn monocytes in culture fail to produce TNF when stimulated with LPS (30). A defect in neonatal responsiveness to LPS is therefore postulated. In adult listeriosis, LPS appears to augment the production of IFN and TNF. Both of these cytokines may have a role in host defense to Listeria. This defect in the newborn therefore may contribute to their susceptibility to Listeria infection (13, 35).

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