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Separate and Combined Effects of Recombinant Interleukin-1α and Gamma Interferon on Antibacterial Resistance

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Our laboratory has previously reported that administration of murine recombinant interleukin 1α (rIL-1α) substantially enhanced the resistance of mice to *Listeria monocytogenes* infection. Other investigators have reported that gamma interferon (IFN-γ) plays a pivotal role in antilisteria resistance. In the present study, we have defined doses of human rIL-1α that enhanced the antilisteria resistance of mice. We then addressed the possibility that combined immunotherapy with rIL-1α and recombinant IFN-γ (rIFN-γ) might result in an additive or synergistic enhancement of antibacterial resistance. Simultaneous administration of rIL-1α and rIFN-γ enhanced antilisteria resistance (at 3 days after infection) to a greater extent than did either cytokine alone, although the results did not imply a synergistic action between the two cytokines. Experiments which examined the effects of the timing of cytokine administration indicated that maximal protection was observed when rIL-1α and rIFN-γ were administered together concomitantly with the *L. monocytogenes* challenge. When we compared the separate and combined protective effects of rIL-1α and rIFN-γ throughout the course of a primary *L. monocytogenes* infection, we observed an additive effect of the two cytokines only at 3 days after challenge, the time at which the peak bacterial burden occurs in the spleens and livers of infected mice. Histopathological comparisons of livers and spleens from cytokine-treated and control listeria-infected mice verified that cytokine treatment reduced the severity of tissue damage in cytokine-treated listeria-infected mice. In an attempt to provide a potential mechanism for the protective effects of rIL-1α and rIFN-γ administration, we compared levels of colony-stimulating activity in sera from cytokine-treated and control listeria-infected mice. The highest levels of colony-stimulating activity were detected in sera from control listeria-infected mice; somewhat lower levels were found in sera from listeria-infected mice that received rIL-1α and rIFN-γ either alone or in combination.

Although the potential importance of cytokines as mediators of host defense against microbial invasion has been postulated for several years, it is only recently that sufficient quantities of recombinant cytokines have been available to allow their therapeutic potential to be assessed. The potential benefits of cytokine administration are particularly attractive as supplementary therapy of antibiotic-resistant bacterial infections and as prophylactic treatment to prevent or reduce the severity of opportunistic infections in immunocompromised hosts.

Previous examinations of the potentiation of antimicrobial resistance by recombinant interleukin-1α (rIL-1α) and recombinant gamma interferon (rIFN-γ) (4, 5, 13, 22) have yielded encouraging results. Studies from this laboratory demonstrated that murine rIL-1α protected mice against infection with *Listeria monocytogenes* (4, 5); other investigators reported similar benefits for human rIL-1α treatment of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* infections in mice (22). Kiderlen et al. (13) reported that administration of high doses (10^6 U per mouse) of rIFN-γ reduced the severity of *L. monocytogenes* infection in mice. Several recent reports have suggested that there may be additive or synergistic effects induced by certain combinations of cytokines (19, 21). These latter studies are of particular relevance to the eventual clinical use of cytokines, since it is likely that the greatest degree of protection, with the least amount of deleterious side effects, may be obtained by the administration of cytokine cocktails consisting of two or more cytokines with complementary or synergistic biological effects. In this study, our purpose was twofold: (i) to examine the dose-response effects of human rIL-1α and murine rIFN-γ on antilisteria resistance, thereby defining optimal and suboptimal doses for both cytokines; and (ii) to determine whether combined therapy of a wide dose range of rIL-1α with a wide dose range of rIFN-γ would have additive or synergistic effects on antilisteria resistance.

MATERIALS AND METHODS

Cytokines. rIL-1α (lot SM 59; Hoffman-La Roche Inc., Nutley, N.J.) was the generous gift of A. Stern and P. T. Lomedico. This material was provided at 1.3×10^6 lymphocyte-activating factor units per ml in 10 mM potassium phosphate buffer and stored at −70°C until used. Appropriate dilutions of rIL-1α were made in pyrogen-free saline that was pH adjusted to 7.2.

Murine rIFN-γ was obtained from two separate sources. The initial experiments that identified and titrated the protective effect of rIFN-γ were performed by using rIFN-γ obtained from Genentech (South San Francisco, Calif.) that was generously provided by G. Byrne (University of Wisconsin, Madison). The majority of the experiments used rIFN-γ donated by the Schering Corporation through the American Cancer Society's program on interferon. In all experiments, appropriate dilutions of rIFN-γ were made in pH-adjusted (7.2) pyrogen-free saline.

Mice. Five- to eight-week-old male (C57BL/6 × DBA/2) F_1 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). These mice were housed and maintained as previously described (4). As stated by the supplier and verified by random health checks in our animal care facility,

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the mice were free of infection by adventitious agents, including Sendai virus and mouse hepatitis virus. These mice were housed at the Charmany Animal Care Facility of the School of Veterinary Medicine.

Bacterial infection. *L. monocytogenes* EGD was maintained as previously described (6). Before each experiment, a sample was thawed and inoculated into tryptone phosphate broth, and the listeriae were grown for 15 to 18 h at 37°C. The bacteria were recovered by centrifugation, washed, and suspended at 2×10^4 viable listeriae in 0.2 ml of pyrogen-free saline. Individual mice were infected intravenously (i.v.) via a lateral tail vein. Unless otherwise stated, the cytokines were injected concomitantly with the listeriae and the mice were killed by cervical dislocation 72 h after infection. Spleens were removed from individual mice, placed into separate sterile glass tissue grinders that contained 5.0 ml of cold phosphate-buffered saline, and homogenized. This time was chosen because it has previously been shown that it coincides with the peak bacterial burden in the spleens of listeria-infected control mice (7). Spleen homogenates were serially diluted in sterile distilled water, and appropriate dilutions were plated on Trypticase soy agar (Difco Laboratories, Detroit, Mich.) The plates were incubated at 37°C for 24 h, at which time the colonies were counted. Results are expressed as either the $\log_{10}$ *L. monocytogenes* (mean ± standard error of the mean [SEM]) per spleen or as the $\log_{10}$ reduction in the numbers of viable *L. monocytogenes* per spleen for cytokine-treated mice compared with those of control listeria-infected mice.

Histopathology. At necropsy, livers and spleens from individual mice were placed into separate vials that contained 10% buffered Formalin. These were embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin by the School of Veterinary Medicine Histopathology Laboratory.

Bioassay for CSA. Sera from control or cytokine-treated listeria-infected mice were assayed for the presence of colony-stimulating activity (CSA) in a soft agar bone marrow culture as previously described (5). Bone marrow cells were collected from the femurs of C57BL/6 mice (Jackson Laboratory) into modified McCoy 5A medium (GIBCO Laboratories, Grand Island, N.Y.), monodispersed, and counted by trypan blue exclusion (viability always >95%). Bone marrow cells were plated at a concentration of 7.5×10^4 cells per ml in a solution containing modified McCoy 5A medium, 20% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), 10^{-4} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.), and 0.3% Bacto-Agar (Difco). The cell suspension was plated at a volume of 1 ml in 35-mm-diameter Falcon petri dishes (Becton Dickinson Labware, Oxnard, Calif.) to which had been added 100 μl of a 1:3 dilution of test serum. WEHI conditioned medium (a source of IL-3) and preimmune serum served as positive and negative controls, respectively. Dishes were prepared in quadruplicate and incubated at 37°C and in 7% CO_2 in a fully humidified incubator for 7 days. Colonies were subsequently stained with luxol fast blue-hematoxylin. Granulocyte-macrophage colony-forming units were defined as aggregates of ≥50 cells.

Statistical analysis. Results were analyzed for statistical significance by a one-way analysis of variance using the BMDP statistical software package (University of California Press, Berkeley, Calif.). Relevant comparisons between groups were made by Student *t* tests. The level of significance for all comparisons was set at $P < 0.05$.

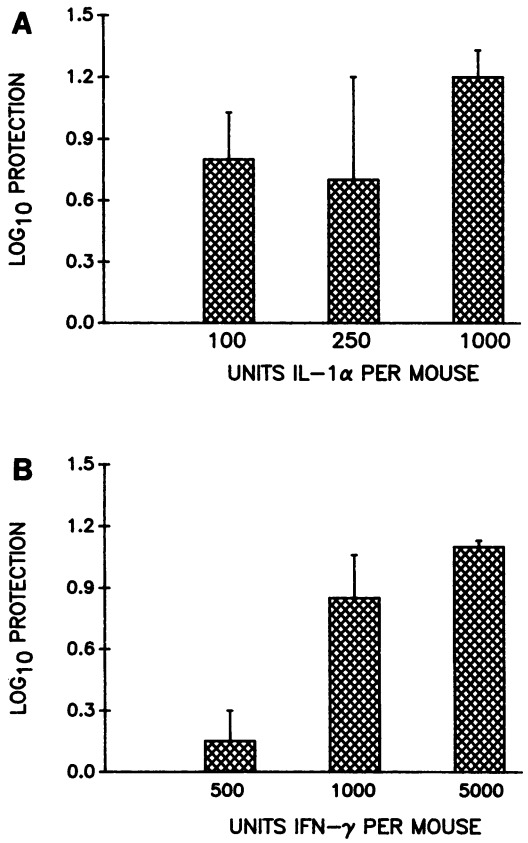

FIG. 1. Dose-response effects of rIL-1α (A) and rIFN-γ (B) on antilisteria resistance. Mice received the indicated doses of rIL-α or rIFN-γ i.v. concomitantly with a sublethal challenge of *L. monocytogenes*. Mice were sacrificed 72 h postchallenge, and the severity of infection was quantitated by recovery of viable listeriae from the spleen. Results are expressed as the $\log_{10}$ protection (mean ± SEM) compared with the numbers of listeriae recovered from the spleens of control listeria-infected mice. The data from this graph were derived from four (100 U of IL-1α), two (250 U of IL-1γ), nine (1,000 U of rIL-1α), two (500 U of rIFN-γ), four (1,000 U of rIFN-γ), and four (5,000 U of rIFN-α) separate experiments, with four mice per experimental group.

RESULTS

Effects of human rIL-1α on antilisteria resistance. It was important to perform dose-response experiments with human rIL-1α to define optimal and suboptimal dosages which protected mice against a sublethal *L. monocytogenes* challenge, since previous results from this laboratory were obtained by using murine rIL-1α. Human rIL-1α significantly augmented antilisteria resistance (Fig. 1A). The maximal level of protection was observed at a dose of 1,000 U (approximately 0.2 μg) of rIL-1α per mouse, which resulted in a 1.2 $\log_{10}$ average reduction in the number of listeriae per spleen at 72 h after infection (nine separate experiments, with four mice per treatment group in each experiment). Significant ($P < 0.05$), but less dramatic, protection was observed after administration of as little as 100 U of rIL-1α per mouse; in a limited number of experiments, intermediate doses (250 to 750 U) of rIL-1α also enhanced protection. Within individual experiments, dose-dependent responses similar to those demonstrated in Fig. 1 were observed (data not shown).

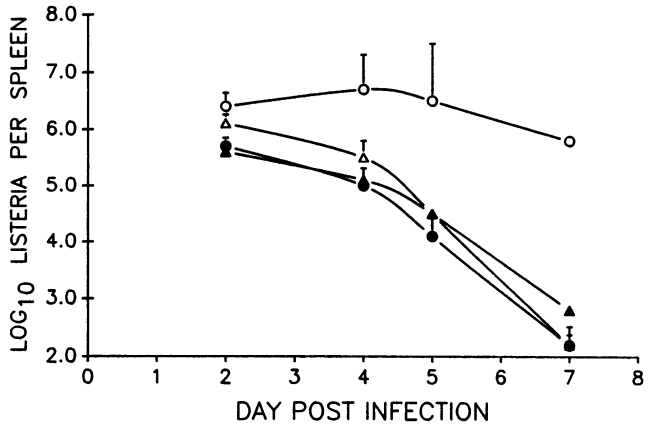

FIG. 2. Separate and combined effects of rIL-1α and rIFN-γ on the course of a primary *L. monocytogenes* infection. Mice received saline (○), 1,000 U of rIL-1α (●), 5,000 U of rIFN-γ (△), or 1,000 U of rIL-1α and 5,000 U of rIFN-γ (▲) concomitantly with an i.v. injection of 2×10^4 *L. monocytogenes*. Mice were sacrificed at the indicated times postchallenge, and the numbers of viable listeriae recovered from the spleens were determined. Results are expressed as the $\log_{10}$ *L. monocytogenes* per spleen (mean ± SEM) (four mice per group).

Effects of murine rIFN-γ on antilisteria resistance. rIFN-γ, like rIL-1α, enhanced antilisteria resistance when injected concomitantly with a sublethal challenge of *L. monocytogenes* (Fig. 1B). At 72 h postchallenge, there was a significant reduction in the numbers of *L. monocytogenes* in the spleens of mice that had received 5,000 or 1,000 U, but not 500 U, of rIFN-γ compared with those of control listeria-infected mice. In a single experiment in which mice received 10,000 U of IFN-γ, we observed a maximal level of protection of approximately 2.0 $\log_{10}$ (4.7 $\log_{10}$ viable *L. monocytogenes* per spleen compared with 6.7 $\log_{10}$ for control mice).

Effects of rIL-1α and rIFN-γ on resistance to listeria infections. We next addressed the question of whether rIL-1α and rIFN-γ, when given simultaneously, had any synergistic or additive protective effect on antilisteria resistance. Various combinations of rIL-1α (100 to 1,000 U) and rIFN-γ (500 to 5,000 U) were injected concomitantly with a sublethal listeria challenge. Table 1 shows the results of three separate experiments in which the combined administration of 1,000 U of rIL-1α and 5,000 U of rIFN-γ resulted in modest but significant ($P < 0.05$) protection that was greater than that which resulted from injection of rIL-1α or rIFN-γ alone.

Timing of rIFN-γ administration in relation to rIL-1α and *L. monocytogenes*. Next, we examined whether it would be possible to improve the additive protective effects of IL-1 and IFN-γ by changing the relative timing of rIFN-γ administration. Results from this experiment (Table 2) demonstrated that simultaneous administration of the two cytokines was the only regimen which resulted in an additive enhancement of antilisteria resistance; administration of rIFN-γ 24 h before or after concomitant injection of rIL-1α and *L. monocytogenes* had no effect on resistance beyond that elicited by rIL-1α alone.

TABLE 1. Separate and combined effects of rIL-1α and rIFN-γ on antilisteria resistance

rIL-1α[a]	rIFN-γ[a]	$\log_{10}$ *L. monocytogenes*/spleen[b]		
		Expt 1	Expt 2	Expt 3
−	−	6.5 ± 0.2	6.5 ± 0.2	7.0 ± 0.2
+	−	5.1 ± 0.4	5.2 ± 0.4	5.3 ± 0.2
−	+	5.4 ± 0.2	5.4 ± 0.2	5.8 ± 0.4
+	+	4.8 ± 0.2	4.5 ± 0.1	4.9 ± 0.4

[a] Mice received 1,000 U of rIL-1α or 5,000 U of rIFN-γ concomitantly with the i.v. injection of 2×10^4 *L. monocytogenes*.
[b] Mean ± SEM (four mice per group).

TABLE 2. Effects of varying time of administration of rIFN-γ on antilisteria resistance of IL-1-treated mice

Treatment[a]	$\log_{10}$ *L. monocytogenes*/spleen[b]
None (control)	7.0 ± 0.1
rIL-1α (day 0)	5.3 ± 0.1
rIFN-γ (day 0)	5.8 ± 0.2
rIL-1α (day 0) + rIFN-γ (day 0)	4.9 ± 0.2
rIL-1α (day 0) + PFS (day −1)	5.6 ± 0.2
rIL-1α (day 0) + rIFN-γ (day −1)	5.3 ± 0.1
rIL-1α (day 0) + PFS (day +1)	5.2 ± 0.1
rIL-1α (day 0) + rIFN-γ (day +1)	5.3 ± 0.2

[a] rIL-1α was administered i.v. at 1,000 U per mouse, and rIFN-γ was administered i.v. at 5,000 U per mouse. Control mice were injected i.v. with an equal volume of pyrogen-free saline (PFS). All mice were challenged with an i.v. injection of 2×10^4 *L. monocytogenes* on day 0.
[b] Mean ± SEM (four mice per group).

Time course of the protective effects of rIL-1α and rIFN-γ during listeria infection. Next, we examined the combined and separate effects of rIFN-γ and rIL-1α on the course of a primary *L. monocytogenes* infection. Mice were injected with *L. monocytogenes* alone, *L. monocytogenes* and 1,000 U of rIL-1α, *L. monocytogenes* and 5,000 U of rIFN-γ, or *L. monocytogenes* with both 1,000 U of rIL-1α and 5,000 U of rIFN-γ. Four mice from each group were sacrificed, and the viable listeriae per spleen were enumerated at 2, 4, 5, and 7 days after infection. The results presented in Fig. 2 demonstrate that at the time points examined in this experiment, 1,000 U of rIL-1α alone had a greater protective effect than did 5,000 U of rIFN-γ alone and that combined administration of rIL-1α and rIFN-γ did not have any benefits beyond that resulting from injection of rIL-1α alone. The apparent discrepancy between these data and those presented in Table 1 may be explained by the fact that the latter were obtained only from mice sacrificed at 3 days postchallenge, the time of peak bacterial burden. In both experimental designs, the effects of rIL-1α and rIFN-γ alone were comparable.

Bioassay for CSA. Previous reports have suggested a possible relationship between serum CSA and antilisteria resistance (29). CSA in sera from cytokine-treated and control listeria-infected mice are presented in Table 3. On each day tested, the highest levels of CSA were observed in sera from control listeria-infected mice; sera from rIL-1α- and rIFN-γ-treated mice had comparable levels of activity which, at days 2 and 7, were significantly higher ($P < 0.05$) than those in sera from mice that had received both rIL-1α and rIFN-γ. In all treatment groups, serum CSA activity was highest early in the infection (day 2) and remained elevated for at least 5 days after bacterial challenge. Because eosinophil colonies were never observed, it is likely that the macrophage and granulocyte colonies formed in response to combinations of granulocyte colony-stimulating factor (CSF) and CSF-1, rather than to high levels of granulocyte-macrophage CSF (18) or multi-CSF (IL-3). This conclusion is

TABLE 3. CSA activity in sera from control and cytokine-treated listeria-infected mice[a]

Day postinfection	CFU-GM[b] (Mean ± SD)			
	Control	rIL-1α	rIFN-γ	rIL-1α + rIFN-γ
2	131 ± 10	90 ± 20	103 ± 19	68 ± 12
4	86 ± 8	43 ± 10	61 ± 22	44 ± 17
5	91 ± 17	46 ± 9	43 ± 7	47 ± 7
7	ND[c]	40 ± 5	22 ± 7	16 ± 6

[a] Mice received 1,000 U of rIL-1α, 5,000 U of rIFN-γ, or 1,000 U of rIL-1α and 5,000 U of rIFN-γ concomitantly with an i.v. injection of 2×10^4 *L. monocytogenes*. Control mice received only listeria organisms.

[b] Granulocyte-macrophage colonies (CFU-GM) formed per 7.5×10^4 bone marrow cells incubated with a 1:3 dilution of serum (four replicate culture dishes for each data point). Results with WEHI conditioned medium and preimmune serum were 128 ± 18 and 11 ± 4, respectively.

[c] ND, Not determined.

supported by a recent report of production of various types of CSF during *L. monocytogenes* infection (3).

Histopathology. Histopathological examination of the livers of cytokine-treated and control listeria-infected mice confirmed our bacteriological evidence for reduction in the severity of infection after administration of rIL-1α or rIFN-γ. The livers of control listeria-infected mice displayed numerous large foci of coagulative necrosis and diffuse Kupffer cell hyperplasia (Fig. 3A). In contrast, tissue sections from cytokine-treated listeria-infected mice exhibited minor histopathologic changes (Fig. 3B), whether the mice received rIL-1α or rIFN-γ alone or in combination.

DISCUSSION

In previous reports from this laboratory (4, 5), we demonstrated that murine rIL-1α, when administered i.v. at a dose of 1,000 U per mouse concomitantly with a sublethal *L. monocytogenes* challenge, significantly enhanced antilisteria resistance. In the present report, we repeated dose-reponse experiments with human IL-1α and found similar protective activity. These experiments were necessary to define optimal and suboptimal ranges that would be used for experiments addressing the combined activities of IL-1 with other cytokines.

In this report, we also confirmed that murine rIFN-γ, when administered at doses of 1,000 to 10,000 U per mouse, significantly enhanced antilisteria resistance. The ability of rIFN-γ to enhance antilisteria resistance has been a subject of considerable discussion. Although Buchmeir and Schreiber (2) reported that in vivo administration of monoclonal anti-IFN-γ antibodies blocked the resolution of *L. monocytogenes* infection in mice and Kiderlen et al. (13) reported that administration of high doses of rIFN-γ (10^6 U per mouse) resulted in a 1.0 $\log_{10}$ reduction in the numbers of *L. monocytogenes* recovered from the spleens of mice 48 h after infection, van Dissel et al. (27) were unable to demonstrate enhanced antilisteria resistance at 48 h after infection in mice that received two i.v. injections of up to 5×10^4 U of rIFN-γ. Our examination of the kinetics of rIFN-γ-mediated enhancement of antilisteria resistance may provide a potential explanation for the discrepancies among these previous reports. We did not detect significant protection after administration of rIFN-γ until 3 days postinfection (Fig. 2). It, therefore, is possible that the inability of van Dissel et al. (27) to demonstrate protection with rIFN-γ and the requirement for extremely high doses of rIFN-γ in the study of Kiderlen et al. (13) may have resulted because they did not examine mice at times later than 48 h postinfection.

At this point, it is difficult to offer a mechanistic explanation for the mode of action of either rIL-1α or rIFN-γ in antilisteria resistance. Intuitively, one would expect that if IL-1α and IFN-γ were acting along different pathways, then the combined administration of rIL-1α and rIFN-γ might result in an additive or synergistic increase in antilisteria resistance. We attempted to examine this possibility in two different sets of experiments. First, we performed checkerboard experiments in which the doses of rIL-1α and rIFN-γ were varied independently but administered simultaneously. Second, using optimal doses of rIL-1α and rIFN-γ, we staggered the administration of rIFN-γ in relation to the injection of rIL-1α. We found that only under limited conditions was it possible to improve the level of protection afforded by administration of optimal doses of either cytokine alone. Although there was a small but consistent

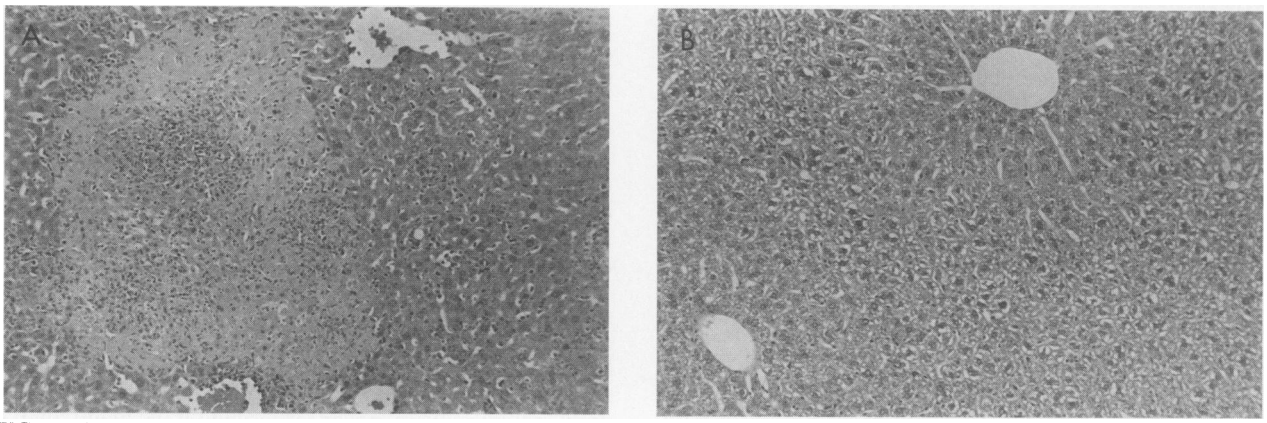

FIG. 3. Comparison of histopathological changes in the livers of cytokine-treated and control listeria-infected mice at 5 days after challenge with 2×10^4 *L. monocytogenes*. Livers of control listeria-infected mice exhibited extensive areas of coagulative necrosis and Kupffer cell hyperplasia (A), whereas in contrast, livers from mice that received 1,000 U of rIL-1α and 5,000 U of rIFN-γ exhibited minor histopathological changes (B). A similar lack of histopathological damage to the liver was noted for mice that received 1,000 U of rIL-1α or 5,000 U of rIFN-γ alone (results not shown).

increase in protection at 3 days postinfection after simultaneous combined administration of IL-1α and IFN-γ, our data did not provide convincing evidence that rIL-1α and rIFN-γ acted along independent pathways.

By surmising from the abundance of information recently published on the pluripotent effects of IL-1 and other cytokines, it is likely that in vivo administration of rIL-1α and rIFN-γ sets into motion a cascade of immunoregulatory events, including the induction of other cytokines. These secondarily induced factors and their effects may then perform important roles in determining the level of antibacterial resistance. For example, IL-1 is known to induce production and release of tumor necrosis factor (TNF) (15, 20, 24), CSF (9, 25, 28), and IL-2 (16) in vivo and IFN-γ in vitro (28). Several recent reports have provided evidence for the participation of these cytokines either singly or in combination in various biological responses that might contribute to host defense against *L. monocytogenes* and other microbial pathogens. For instance, Havell (10) has demonstrated that administration of anti-TNF antibody into sublethally infected mice abrogated antilisteria resistance. Other investigators have shown that TNF alone (20) or acting synergistically with rIFN-γ can enhance macrophage killing of some types of parasites (8). Another example is that of the CSFs, where granulocyte-macrophage CSF has been shown to enhance the antimicrobial activity of mature phagocytes (18), CSF-1 has been shown to augment macrophage oxidative activity (30), and granulocyte CSF has been shown to protect neutropenic mice from infection with several bacteria and *Candida albicans* (17). Likewise, interferons which are released during infection with *L. monocytogenes* may also modulate antilisteria resistance (11). IFN-γ increases the expression of TNF receptors (26) and Ia on macrophages (1, 31), both of which might lead to more efficient interaction between macrophages and listeria-specific T cells. These interactions might indirectly up-regulate the T-cell activation (12) that is clearly required for release of additional IFN-γ and perhaps other macrophage-activating factors that may augment the destruction of intracellular listeria by macrophages (14, 23).

In this study, we attempted to assess the relative levels of some of the aforementioned cytokines in sera from cytokine-treated and control listeria-infected mice. We were particularly interested in determining relative levels of CSA in sera because an apparent relationship between CSA and expression of antilisteria resistance has been reported (29, 32). Control listeria-infected mouse sera had the highest levels of CSA, whereas sera from mice that received cytokine therapy had lower levels of CSA. At days 2 and 7 postinjection, sera from mice that received combined therapy had lower levels of CSA than did sera from mice that received either cytokine alone. It is difficult to explain these results. Perhaps a detailed examination of time points earlier than day 2 postinjection would illuminate mechanism(s) underlying this phenomenon. These experiments are under consideration. We have also attempted to measure serum IFN and TNF levels in listeria-infected mice. We have been unsuccessful in measuring the low levels of IFN likely to be present in mouse sera during listeria infection. In addition, we can confirm the report of Havell (10) that TNF activity is not present in the sera of mice undergoing a sublethal *L. monocytogenes* infection if they are not challenged with lipopolysaccharide (data not shown).

In summary, we have defined the doses of exogenously administered rIL-1α or rIFN-γ that significantly enhance antilisteria resistance in mice. A slight, but consistent and significant ($P < 0.05$), enhancement of antilisteria resistance over that induced by either cytokine alone was observed 3 days after infection of mice that had received rIL-1α and rIFN-γ in combination at the time of the *L. monocytogenes* challenge. Our results and those of other investigators suggest that cytokine therapy may have therapeutic potential for reducing the morbidity and mortality of bacterial infections. Use of these immunoregulatory agents may be particularly attractive for combating the problem of opportunistic infections in immunocompromised hosts. Because the greatest therapeutic success ultimately may be achieved by administration of combinations of several cytokines, it will be important to evaluate the level of antibacterial resistance afforded by candidate cytokines both singly and in various combinations. Further examination of the modes of action of these cytokines in experimental infection models, such as the one described here, are likely to provide additional insight into the complex mechanisms of immunoregulation of antibacterial resistance.

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