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Evidence that Endogenous Gamma Interferon Is Produced Early in *Listeria monocytogenes* Infection

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It has been presumed that gamma interferon (IFN-γ), which plays an essential role in antilisterial resistance, is produced late in *Listeria monocytogenes* infection. In the present study, however, IFN-γ was detected in the bloodstreams and spleens of mice from days 1 to 4 of *L. monocytogenes* infection by both a double-sandwich enzyme-linked immunosorbent assay and an immunohistochemical technique, suggesting that endogenous IFN-γ is produced early but not late in *L. monocytogenes* infection.

Complete elimination of *Listeria monocytogenes*, a facultative intracellular pathogen, from the tissues of infected animals is performed by macrophages activated by T-cell-dependent mechanisms (15, 16, 21). Cytokines, which are produced mainly by immunocompetent cells, play crucial roles in the establishment and expression of various immune responses. The essential role of gamma interferon (IFN-γ) in antilisterial resistance has been demonstrated by in vivo injections of antibodies against this factor (1, 2, 17) or recombinant IFN-γ (12). Endogenous IFN-γ in the bloodstreams of *L. monocytogenes*-infected mice could not be detected unless the mice were stimulated with specific antigen or reinfected after 4 days of infection (17). IFN-γ production induced by specific antigen in mouse spleen cell cultures progresses with similar kinetics after *L. monocytogenes* infection (2, 10, 11, 13). In a recent report (5), it was also demonstrated that the numbers of IFN-γ-producing cells in spleens detected by a solid-phase immunoenzymatic technique began to rise on day 4 and attained a maximum on days 7 and 8 of infection. However, in a previous study (19) we demonstrated that antilisterial resistance is suppressed when a single injection of anti-mouse IFN-γ monoclonal antibody (MAb) is given before day 2 of infection, suggesting that endogenous IFN-γ might be produced not late but early in infection. Therefore, we focused our studies on determination of when endogenous IFN-γ would be produced in the course of *L. monocytogenes* infection. In this report, we provide evidence that endogenous IFN-γ is persistently detected in the bloodstreams and spleens of mice from days 1 to 4 of infection, but not thereafter.

Female ddY outbred mice (age, 5 to 7 weeks; obtained from SLC, Hamamatsu, Shizuoka, Japan) were used. Serum samples and spleen extracts were obtained from mice at progressive times over a 14-day period after intravenous infection with 2×10^4 CFU of viable *L. monocytogenes* cells in 0.9% physiological saline. Extracts of spleen homogenate were prepared as follows. The spleens were aseptically removed from the mice and suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.), and 10% (wt/vol) homogenates were prepared with a Dounce tissue grinder. The homogenates were frozen, then thawed twice, and clarified by centrifugation at $2,000 \times g$ for 10 min. The extracts that were obtained were assayed for IFN-γ. The IFN-γ assay was carried out by a double-sandwich enzyme-linked immunosorbent assay (ELISA) developed by Curry et al. (4). Purified rat anti-mouse IFN-γ MAb was used as a primary antibody. The MAb was obtained from the ascites fluid in pristane-primed BALB/c nude mice injected with hybridoma R4-6A2 (7) and purified by DEAE Affi-Gel Blue column chromatography as described previously (19). Rabbit anti-mouse IFN-γ serum was used as a secondary antibody. The immune serum was obtained from a rabbit that was hyperimmunized with purified recombinant mouse IFN-γ as described previously (4). All ELISAs were run with recombinant mouse IFN-γ that was produced and purified by Genentech, Inc. (San Francisco, Calif.); this IFN-γ was already titrated by antiviral assays and was used as a standard. The specificity and sensitivity (as little as 0.1 antiviral IU/ml of mouse IFN-γ) of the ELISA used in this study were the same as those reported by Curry et al. (4).

IFN-γ in both the sera and the spleen extracts was assayed by ELISA at various times following *L. monocytogenes* infection (Fig. 1). IFN-γ appeared in spleen extracts 16 h after infection, appeared in the bloodstream 24 h after infection, and then peaked at 48 h. IFN-γ could be detected until day 4 of infection, but not thereafter. To investigate whether endogenous IFN-γ detected by ELISA would be biologically active, antiviral activities of the spleen extracts were determined by a bioassay (Table 1). The bioassay was carried out by the dye-binding method with mouse L-929 cells and vesicular stomatitis virus (Indiana strain) as described previously (17). In previous studies (17, 18, 20), we demonstrated that IFN-α is also detected in the bloodstreams and spleens of mice on days 1 to 3 of *L. monocytogenes* infection. IFN-α and IFN-γ were distinguished by neutralization tests by using each MAb (Table 1). Samples that were serially diluted twofold were mixed with equal volumes of anti-mouse IFN-α MAb, anti-mouse IFN-γ MAb, or both. The mixtures were incubated at 37°C for 1 h before they were assayed for antiviral activities. The antiviral activities of all specimens that we used were partially but significantly decreased after treatment with MAbs against mouse IFN-α or mouse IFN-γ and were neutralized completely with the mixed MAbs. After treatment with anti-mouse IFN-α MAb, i.e., IFN-γ, the IFN titers were almost comparable to those obtained by ELISA, suggesting that the IFN-γ detected by ELISA was biologically active.

We also identified endogenous IFN-γ in the spleens of *L. monocytogenes*-infected mice by an immunohistochemical technique. Briefly, blocks of the spleens were oriented

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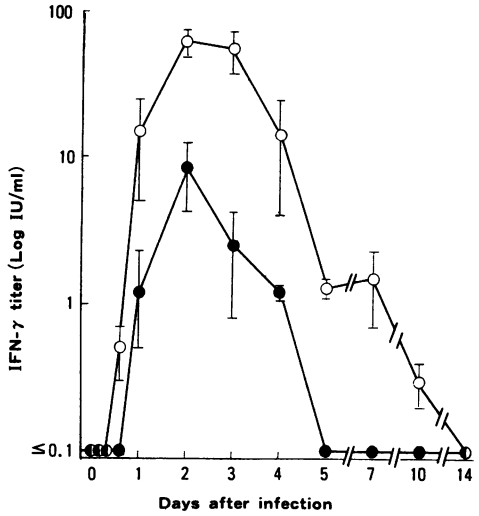

FIG. 1. Production of IFN-γ in the bloodstreams and spleens of mice following *L. monocytogenes* infection. IFN-γ in sera (●) and spleen extracts (○) was determined by ELISA. Each point represents the mean ± standard deviation for a group of five mice.

frozen in OCT compound (Ames Co., Div. of Miles Laboratory, Inc., Elkhart, Ind.) and stored at −70°C. Acetone-fixed frozen tissue sections (thickness, 4 μm) were stained by a sensitive immunoperoxidase technique by using the avidin-biotin complex method (6). This involves successive incubations with normal rabbit serum for 15 min and optimal dilutions of rat MAbs against mouse IFN-α, mouse IFN-β, mouse IFN-γ, or normal rat immunoglobulin G (IgG) for 60 min; biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, Calif.) for 30 min; and then avidin-biotinylated peroxidase complex (Vector Laboratories) for 30 min. The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in phosphate-buffered saline for 15 min before the avidin-biotin-peroxidase step. The sections were stained by incubation in a solution of 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Inc., Milwaukee, Wis.). They were counterstained with hematoxylin and mounted in phosphate-buffered saline–glycerol.

The frozen sections of spleens obtained from *L. monocytogenes*-infected mice 4, 8, 24, and 48 h after infection or from uninfected animals were stained with MAbs against the three types of mouse IFNs used in this study or normal rat IgG. No staining was seen in control slides from any specimens treated with normal rat IgG (data not shown) or anti-mouse IFN-β MAb (Fig. 2A) as the primary antibody. In contrast, IFN-γ could be detected in the spleen extracts obtained from mice 24 and 48 h after infection (Fig. 2B). The frozen sections also showed immunoreactivity with anti-mouse IFN-α MAb (Fig. 2C). IFN-α was detected 4 h after infection (data not shown).

It has been presumed that IFN-γ should be produced by sensitized T cells late in *L. monocytogenes* infection (2, 5, 10, 11, 13, 17). However, there may be some doubts about

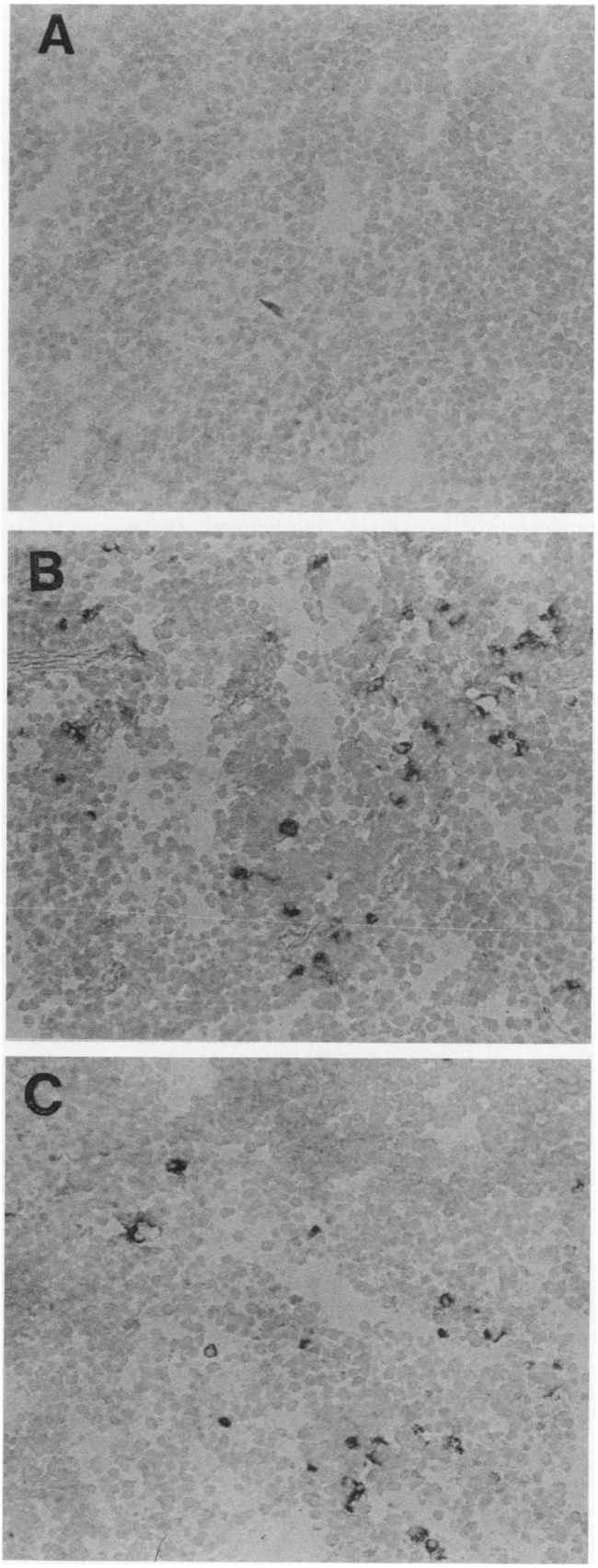

FIG. 2. Detection of endogenous IFNs in the spleens of *L. monocytogenes*-infected mice by an immunohistochemical technique. Frozen tissue sections of the spleens obtained from mice 48 h after infection were stained with MAb against mouse IFN-β (A), mouse IFN-γ (B), or mouse IFN-α (C). Magnifications, ×186.

TABLE 1. Antiviral activity and antigenicity of IFN-γ detected in spleen extracts of *L. monocytogenes*-infected mice

Spleen extract or control[a]	IFN-γ titer (IU/ml) by ELISA	IFN titer (IU/ml) by the bioassay after treatment with[b]:			
		Not treated	Anti-IFN-α	Anti-IFN-γ	Anti-IFN-α + anti-IFN-γ
Day 1	32	55	26	28	<8
Day 2	121	236	92	57	<8
Day 3	64	64	33	12	<8
Day 4	50	64	43	18	<8
Recombinant mouse IFN-γ	68	64	61	<4	<4
Natural mouse IFN-α/β	<0.1	320	82	314	78

[a] Mice were infected intravenously with 2×10^4 CFU of *L. monocytogenes* cells.
[b] The neutralization test was carried out as described in the text.

this presumption. For instance, estimation of IFN-γ induced by stimulation with specific antigen, that is, the productivity of IFN-γ, might not necessarily coincide with endogenous production of the cytokine. Furthermore, endogenous IFN-γ should be produced early in infection, as judged by the possible roles of IFN-γ in antilisterial resistance. The significant elimination of *L. monocytogenes* cells from the organs of infected mice has been observed from day 3 of infection (15). In this study we demonstrated by ELISA and the immunohistochemical technique (Fig. 1 and 2) that endogenous IFN-γ would be produced early in *L. monocytogenes* infection. We failed to detect endogenous IFN-γ in the bloodstreams of mice during *L. monocytogenes* infection by the bioassay. IFN-α and IFN-γ are produced endogenously with almost the same kinetics; that is, they appear in the bloodstream 24 h after infection, peak at 48 h, and decline thereafter (Fig. 1) (17). The titers of IFN-α are considerably higher than those of IFN-γ in the bloodstreams of infected mice (IFN-α versus IFN-γ, 60 to 220 IU/ml versus 2 to 13 IU/ml 48 h after infection, respectively). Therefore, IFN-γ would be masked behind IFN-α. Although we could not confirm the antiviral activity of IFN-γ in serum because of its lower titer, IFN-γ in the spleen extracts was biologically active (Table 1).

In a previous study (19), we showed that endogenous IFN-γ produced early in infection might play crucial roles in both nonspecific and acquired antilisterial resistance by in vivo injection of anti-mouse IFN-γ MAb. Other cytokines involving tumor necrosis factor (8, 9, 18), interleukin-1 (14), and colony-stimulating factors (3) may also be produced endogenously in mice with *L. monocytogenes* infection, and collaboration between IFN-γ and other cytokines would give rise to expression of antilisterial resistance. Hence, we are performing studies to clarify the roles of endogenous IFN-γ in antilisterial resistance from the standpoint of interactions between IFN-γ and other cytokines.

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