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Synthesis and Secretion of Interferon by Murine Fibroblasts in Response to Intracellular *Listeria monocytogenes*

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***Listeria monocytogenes*, a gram-positive facultative intracellular bacterium, was shown to be capable of infecting and proliferating in murine embryo fibroblasts. During exponential proliferation, the doubling time of the bacterium was determined to be 2.5 h intracellularly, compared with 25 min extracellularly. Progressive intracellular growth of listeriae ultimately resulted in the destruction of initially infected cells and the spread of infection to neighboring cells. *Listeria* infection induced fibroblasts to synthesize considerable quantities of an acid-stable interferon that proved to be antigenically indistinguishable from both polyinosinic-polycytidylic acid-induced and virus-induced interferon.**

All three antigenically distinct interferon (IFN) classes (IFN-α, IFN-β, and IFN-γ) are produced by mice during a sublethal immunizing infection with *Listeria monocytogenes* (2, 5, 16, 17). One day after an intravenous inoculation of listeriae, IFN-α and IFN-β (IFN-α/β) are found in the serum. Peak serum titers are reached on or about day 2, when the highest numbers of listeriae are present in the liver and spleen (5, 14, 16, 17). At this stage, histological examination of infected livers reveals that infective foci consist of heavily parasitized parenchymal cells surrounded by parasitized neutrophils and macrophages (18). The presence of bacteria in nonprofessional phagocytes that probably lack the microbicidal mechanisms needed to destroy internalized bacteria could constitute an important aspect in the pathogenesis of listeriosis and other infections. Obviously, there must be a mechanism capable of controlling the intracellular proliferation of this bacterium to some degree in nonphagocytic cells, or there would be overwhelming bacterial multiplication and the uncontrolled spread of infection to other nonprofessional phagocytes before specific immunity can be generated.

IFNs inhibit the replication of virus as well as the intracellular multiplication of a variety of nonviral parasites (20, 22), including bacteria (4, 13, 21, 25). There is a need, therefore, to determine whether *Listeria*-induced IFN-α/β plays a role in protecting nonprofessional phagocytes as well as professional phagocytes from unrestricted intracellular *Listeria* multiplication.

In this paper is described an in vitro model of *L. monocytogenes* infection of murine embryo fibroblasts (MEF). This model was developed to study the nature of *Listeria* infection and, eventually, the effects of IFNs on infection and intracellular proliferation of this facultative intracellular bacterium in a nonprofessional phagocytic cell. The model uses the aminoglycoside antibiotic gentamicin sulfate at a concentration in culture that is lethal for extracellular but not intracellular listeriae. This system allowed the examination of the ability of listeriae to gain entry and multiply in nonprofessional phagocytes. Moreover, the use of semisolid agarose overlays on infected MEF monolayers allowed the microscopic observation of cell-to-cell spread of infection. Results will also be presented showing that intracellular multiplication of listeriae induces MEF to synthesize appreciable quantities of the antiviral molecules IFN-α/β. This appears to be the first demonstration of IFN induction in a nonprofessional phagocytic cell by a gram-positive bacterium.

MATERIALS AND METHODS

MEF cultures. Primary cultures of MEF cells were established from AB6F1 (A/Tru × C57BL/6Tru) embryos. Six to eight embryos, at 14 to 17 days of gestation, were aseptically removed from the uterus and placed in a 100-mm petri dish containing 10 ml of pH 7.4 phosphate-buffered saline (PBS) and 0.25% trypsin (GIBCO, Grand Island, N.Y.). Heads and appendages were excised and discarded, and the torsos were finely minced with scissors. The tissue mince was placed in a trypsinization flask with an additional 40 ml of trypsin solution and agitated at 37°C. The trypsin digest was then passed through sterile gauze to remove tissue pieces. Cells in the filtrate were pelleted by slow-speed centrifugation, the trypsin solution was decanted, and the cell pellet was suspended in 100 ml of Eagle minimum essential medium (EMEM) (Microbiological Associates, Walkersville, Md.) containing 5% fetal bovine serum (FBS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5 μg of gentamicin sulfate (Schering Corp., Kenilworth, N.J.) per ml. Cell suspension (25 ml) was placed in 75-cm^2 plastic tissue culture flasks, and the flasks were incubated at 37°C in a 5% CO_2 environment. The next day, the culture medium was aspirated, and fresh medium was added to the flasks, which were then incubated until the cells reached confluency. Confluent cells were trypsinized and split 1:3 at each passage. At the third passage, 10^5 cells in 2 ml of medium containing gentamicin sulfate (0.25 μg/ml) as the only antibiotic were placed into 35-mm culture dishes. Confluent MEF monolayers (4×10^5 cells) resulted 3 to 4 days later.

Staining of MEF cultures in 35-mm dishes was carried out by a modified Wright staining procedure with a Diff-Quick Stain set (American Scientific Products, McGraw Park, Ill.).

Infection of MEF. *L. monocytogenes* (serotype 1/2a; 50% lethal dose [LD_{50}], 2×10^4 CFU in AB6F1 mice) was passaged in mice, grown in Trypticase soy broth (BBL Microbiology Systems), dispensed as stock cultures, and stored at −70°C. MEF monolayers were infected by adding 0.5 ml of a suspension of listeriae at a ratio of 10 bacteria per fibroblast. The listeriae were suspended in EMEM containing 5% FBS and gentamicin sulfate (0.25 μg/ml). The MEF cultures were incubated at 37°C for 2 h on a rocking platform to ensure even dispersion of bacteria. Following incubation,

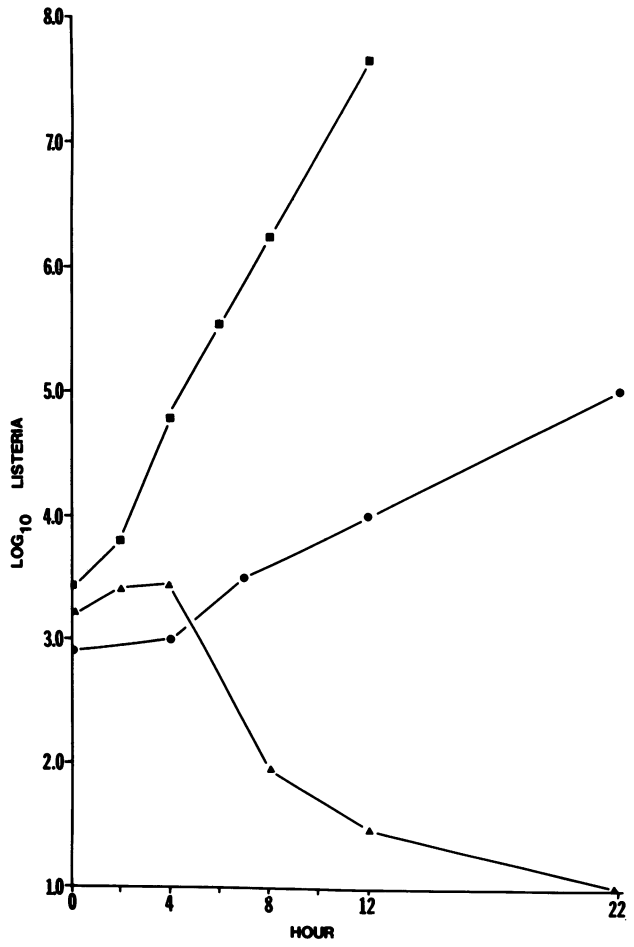

FIG. 1. Intracellular and extracellular multiplication of *L. monocytogenes*. Listeriae were grown in EMEM containing no antibiotics (■), EMEM containing gentamicin sulfate (0.25 μg/ml) (▲), or in MEF monolayers incubated with EMEM containing gentamicin sulfate (0.25 μg/ml) (●).

the medium was aspirated, and the monolayers were washed three times with 1 ml of medium to remove most bacteria not associated with cells. The cultures then received 2 ml of fresh EMEM containing 5% FBS and gentamicin sulfate (0.25 μg/ml). When agarose overlays were used, 2 ml of EMEM containing 1% agarose (SeaKem, Rockland, Maine) 5% FBS, and 0.25 μg of gentamicin sulfate per ml (maintained at 48°C) was added to the MEF cultures following the infection and washing procedures (time zero). After solidification of the overlay, the cultures were inverted and incubated as above.

Enumeration of listeriae. Enumeration of cell-associated listeriae was performed by washing the MEF monolayers three times and then lysing the monolayers with 0.05% deoxycholate (DOC) (Sigma Chemical Co., St. Louis, Mo.). DOC (1 ml) was placed on the monolayers for 5 min at room temperature, after which the dishes were scraped with a rubber policeman, and the cell lysate was placed in a chilled test tube. The culture dishes were washed with an additional 1 ml of DOC, which was then added to the original lysate. The total lysate was then subjected to ultrasound for 10 s to disperse the bacteria. Previous studies established that a 1-h treatment with 0.05% DOC did not affect listerial viability. Lysates were serially diluted in saline (0.85%) and plated on Trypticase-soy agar to enumerate *Listeria* CFU.

IFNs and anti-IFNs. A modified IFN microtiter assay based on that originally reported by Havell and Vilcek (9) with murine L929B cells and vesicular stomatitis virus was used. Included in each IFN assay was an internal laboratory standard which was calibrated to the international murine IFN-α/β standard (G-002-904-511). All IFN titers are corrected to international murine IFN-α/β reference units.

Three murine IFN preparations were used in these studies. A polyinosinic-polycytidylic acid [poly(I)-poly(C)]-induced MEF IFN was prepared by treating confluent 35-mm-dish MEF cultures with 2 ml of poly(I)-poly(C) (10 μg/ml) and DEAE-dextran (100 μg/ml) in PBS for 2 h. After this time, the poly(I)-poly(C) and DEAE-dextran mixture was removed, and the cultures were washed three times and incubated with 2 ml of EMEM containing 5% FBS and antibiotics for 24 h at 37°C. A highly purified preparation (10^8 U/mg of protein) of Newcastle disease virus-induced C243 IFN-α/β (3) was purchased from Enzo Biochem (New York, N.Y.). Phytohemagglutinin (PHA)-induced spleen cell IFN-γ was prepared as described previously (8). Acid treatment of IFN preparations was carried out by dialyzing the IFNs against Clark Lubs HCl-KCl buffer, pH 2.0. After 5 days, the acidified samples were dialyzed against PBS (pH 7.4).

Anti-IFN antibody neutralization assays were performed as described by Havell and Spitalny (6). The sheep anti-murine IFN-α/β neutralizing antibody was a gift from Ion Gresser (Villejuif, France). The monoclonal rat anti-murine IFN-γ immunoglobulin G1 was prepared in this laboratory (23). The neutralizing titer of the antibodies was defined as the reciprocal of the dilution of antibody which, when reacted with an equal volume of IFN (20 U/ml), neutralized 50% of the IFN antiviral activity, as judged by the development of vesicular stomatis virus cytopathic effects in L929B cells.

RESULTS

Intracellular multiplication of *L. monocytogenes* in MEF. To undertake in vitro studies to determine the intracellular fate of *L. monocytogenes* in MEF cells, a technical difficulty had to be overcome: a method was needed to prevent multiplication of extracellular bacteria. It was reasoned that if an antibiotic was included in the MEF culture medium at a concentration that was either listericidal or listeriostatic only for extracellular bacteria, any observed increase in the number of bacteria could be attributed to intracellular multiplication. The broad-spectrum aminoglycoside antibiotic gentamicin sulfate was selected because aminoglycoside antibiotics have relatively little effect on intracellular bacterial proliferation (26) as a result of their inability to effectively penetrate mammalian cells. On the other hand, gentamicin sulfate, even at very low concentrations, was found to kill extracellular listeriae. It was determined that at a concentration of 0.25 μg/ml in culture medium, gentamicin sulfate inhibited listerial growth for the first 4 h and then caused a 10-fold decrease in viability over the next 4 h (Fig. 1). The constant levels of bacteria over the first 4 h could be due to either a bacteriostatic effect or an equilibrium between bacterial growth and death. In the absence of the antibiotic, listeriae proliferated exponentially in culture medium, with a doubling time of approximately 25 min (Fig. 1).

With the knowledge that gentamicin sulfate at 0.25 μg/ml was listericidal for extracellular bacteria, a study was next undertaken to determine whether this concentration of anti-

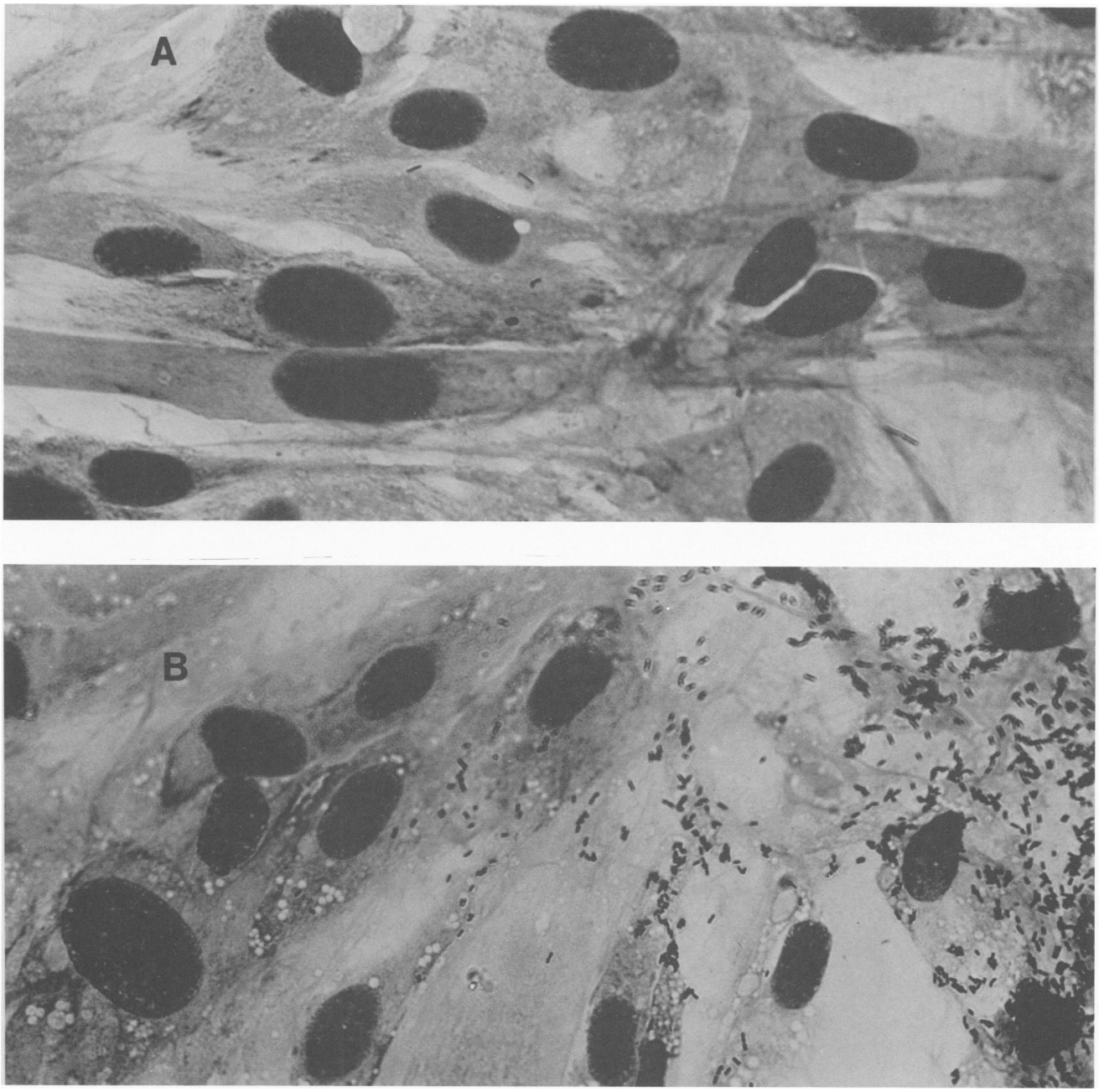

FIG. 2. Course of *L. monocytogenes* infection in MEF. (A) Washed MEF monolayer immediately following the 2-h adsorption period. Several MEF are seen associated with a single bacterium. (B) Listeria-infected MEF maintained for 24 h under a semisolid agarose medium overlay. This field of view shows radial cell-to-cell spread of infection from a central focus of heavily infected MEF. Magnification, ×400.

biotic could affect the viability of listeriae within MEF cells. Replicate cultures of confluent MEF were incubated with listeriae at a ratio of 10 bacteria to 1 MEF for 2 h, and the cultures were then washed to remove listeriae not associated with MEF. Cultures were then replenished with medium containing gentamicin sulfate (0.25 μg/ml) and incubated at 37°C. At the time that culture medium was added (time zero) and at progressive times thereafter, cultures were removed from the incubator, washed, fixed, stained, and examined microscopically. Microscopic examination revealed that at time zero less than 1% of the MEF cells had listeriae associated with them and that the mean number of listeriae per infected MEF was 1. However, as many as three bacteria were seen to be associated with some MEF. There was also considerable variability between experiments in the number of MEF associated with listeriae. For example, the percentage of cells associated with bacteria at time zero ranged from 0.1 to 5% under identical conditions of infection in different studies. In this study, it was found that immediately following the initial infection procedure, approximately 10^3 bacteria were associated with the entire MEF monolayer (2×10^5 cells), and the number of bacteria remained constant for the first 4 h of infection (Fig. 1). After this time, however, the number increased exponentially, doubling every 2.5 h.

L. monocytogenes infection of MEF cultures was also followed microscopically over time. Immediately following

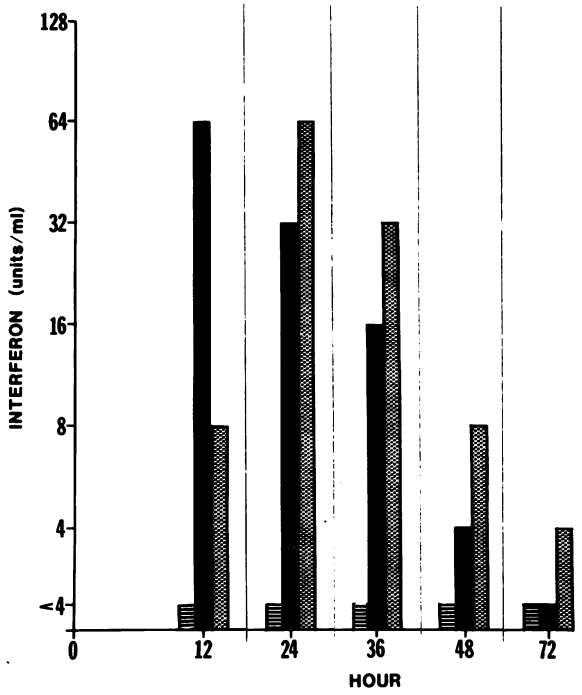

FIG. 3. *L. monocytogenes*-induced IFN synthesis in MEF. Interferon titers in MEF culture media were determined at various intervals following the initial 2-h adsorption. Ratio of infection: 10 listeriae per cell (solid bars); 0.5 listeriae per cell (stippled bars); no listeriae (striped bars).

the adsorption and washing procedure (zero time), very few listeriae were associated with individual cells, even in the selected field shown (Fig. 2A). In fact, most cells in the monolayer were free of listeriae. In contrast, infected MEF cultures incubated for 24 h under a solidified agarose overlay showed multiple foci of infection, each consisting of a central cluster of both dead and living cells heavily laden with listeriae surrounded by cells containing fewer bacteria (Fig. 2B). As pointed out earlier, the function of the agarose overlay was to ensure cell-to-cell infection with listeriae instead of a generalized infection of the MEF monolayer by the release of bacteria from dead cells, and the dispersal of bacteria throughout the liquid culture medium to cells distant from primary foci of infection. In regard to this last point, microscopic examination of infected MEF monolayers maintained in liquid culture medium for a similar length of time (24 h) showed that the number of infected cells was much higher and involved cells scattered throughout the culture. By 48 h almost total destruction of the monolayers had occurred. In contrast, 48-h cultures overlaid with agarose showed the same number of infectious foci as observed 24 h earlier, but each focus was larger and contained more dead cells. The progressive nature of *Listeria* infection of MEF cultures maintained under agarose overlays in the presence of gentamicin sulfate eventually became macroscopically evident as discrete plaques in the MEF monolayers.

Several points of interest concerning the cell-to-cell infection process are evident in Fig. 2B. First, cell-to-cell infection was not contingent on disruption of the infected cell and the subsequent release of listeriae. This can be suggested because even towards the center of the focus of infection, many heavily infected cells had intact plasma membranes. Certainly, cells posessing fewer listeriae towards the periphery of the infectious focus were viable and intact at the time of fixation. Moreover, in these cells, the listeriae appeared to be within phagocytic vacuoles. This would suggest, therefore, that internalization of listeriae may occur through phagocytosis.

***Listeria*-infected MEF synthesize IFN.** A variety of virus-infected murine cells synthesize IFN-α/β during viral replication (12, 27, 28). Therefore, it was of interest to determine whether intracellular listeriae multiplication in MEF also caused the induction of IFN-α/β. To investigate this possibility, replicate MEF cultures were incubated for 2 h with medium containing listeriae at concentrations which gave a multiplicity of infection of either 10 or 0.5 bacteria per cell. Following the 2-h adsorption period, cultures were washed and incubated with 1 ml of medium containing gentamicin sulfate (0.25 μg/ml). At designated times thereafter, media from replicate cultures were pooled, centrifuged to remove bacteria, and assayed for IFN antiviral activities. The experimental cultures were then replenished with fresh medium and incubated until the next designated collection time.

The results of the IFN assays showed unequivocally that MEF produced IFN in response to *Listeria* infection. MEF cells treated with the larger number of listeriae (10 bacteria per cell) synthesized the most IFN (64 U/ml) during the first 12 h of infection (Fig. 3). Thereafter, the production of IFN slowly diminished. The decline in IFN synthesis correlated with the progressive degeneration of MEF monolayers, as assessed microscopically. On the other hand, MEF cultures infected with 20 times fewer listeriae produced only 8 U of IFN per ml during the first 12 h of infection. However, peak IFN synthesis in these cultures occurred over the next 12-h interval, after which the rate of IFN production declined in a manner similar to that observed in more heavily infected cultures. Only viable listeriae induced MEF to synthesize IFN, in that MEF cultures incubated with medium contain-

TABLE 1. Antigenicity and acid stability of *L. monocytogenes*-induced MEF IFN

Cell culture	IFN-inducing agent	IFN (U/ml)[a]		Antibody-neutralizing titer[b]	
		Before	After	Anti-IFN-α/β	Anti-IFN-γ
MEF	None	<4	—[c]	—	—
	Listeriae (viable)	256	256	9,830	<16
	Listeriae (inactivated)[d]	<4	—	—	—
	Poly(I)-poly(C) + DEAE-dextran	8,192	8,192	9,830	<16
C243	Newcastle disease virus	1,024	1,024	6,554	<16
Spleen	PHA	1,024	32[e]	<16	819

[a] Before and after treatment at pH 2.0.

[b] The antibody-neutralizing titer for IFN antiviral activity was defined as the reciprocal of the highest dilution of antibody that, when mixed with an equal volume of IFN (final IFN concentration, 10 U/ml), neutralized 50% of the antiviral activity, as judged by the development of viral cytopathic effect.

[c] —, Not done.

[d] Inactivation of listeriae by UV irradiation was carried out as described previously (5). Confluent MEF cultures were incubated with 2 ml of EMEM containing 5% FBS, gentamicin sulfate (0.25 μg/ml), and 10^7 UV-inactivated listeriae. Two days later, the culture media were collected, pooled, and centrifuged, and the supernatant was assayed for IFN antiviral activity.

[e] The IFN antiviral activity remaining after acidification of PHA-induced spleen cell IFN-γ antiviral activity has been shown through serological analysis to be an acid-stable form of IFN-γ (8).

ing 10^7 UV-inactivated listeriae for 48 h did not synthesize IFN (Table 1).

Properties of *Listeria*-induced MEF IFN. The *Listeria*-induced MEF IFN was characterized for its acid stability and antigenicity. MEF cultures infected with viable listeriae produced an IFN that was stable for 5 days at pH 2.0 (Table 1). Anti-IFN antibody neutralization assays of different MEF IFN preparations with antibodies specific for either IFN-α/β or IFN-γ were also done. The antiviral activity of MEF IFN induced with listeriae or with poly(I)-poly(C) was neutralized to approximately the same degree by the anti-IFN-α/β antibodies (Table 1). In contrast, a monoclonal anti-IFN-γ neutralizing antibody did not neutralize the antiviral activity of the MEF IFN preparations or of Newcastle disease virus C243 cell IFN-α/β. These results establish, therefore, that *Listeria*-induced MEF IFN is antigenically indistinguishable from acid-stable, virus-induced IFN-α/β. Other experiments showed that listeria-induced MEF IFN, like other murine IFNs, did not render human fibroblasts resistant to viral replication (results not shown).

DISCUSSION

The results of the studies presented here establish that MEF can internalize and support the intracellular proliferation of *L. monocytogenes*. The results show, in addition, that during the intracellular multiplication of this gram-positive, facultative intracellular bacterium, IFN-α/β was synthesized and secreted. IFN was initially identified as a product of viral infection, but IFN synthesis can also be induced by certain bacteria. For example, Stinebring and Younger (24) reported that IFN was detectable in the sera of mice shortly after an intravenous injection of gram-negative bacteria, and Ho reported a similar finding for rabbits (10). These and subsequent studies implicated endotoxin as the component of gram-negative bacteria responsible for IFN induction (29, 30), and macrophages were suggested to be the source of the IFN (1, 7). Until now, the only reports of IFN induction by bacteria in cultures of nonlymphoid cells not considered professional phagocytes have dealt with cells infected with *chlamydiae* (11, 15) and rickettsiae (H. E. Hopps, S. Kohno, M. Kohno, and J. E. Smadel, Bacteriol. Proc., p. 115, 1964), bacteria that are gram-negative and, like viruses, are obligate intracellular parasites.

The demonstration here that *L. monocytogenes* multiplies inside MEF is based on the knowledge that the concentration of gentamicin sulfate in the culture medium was listericidal only for extracellular bacteria (Fig. 1). Moreover, microscopic examination of *Listeria*-infected MEF cultures incubated for 48 h under an antibiotic-containing agarose overlay revealed that, regardless of the extent of infection, listeriae were never present outside the cells viewed in profile. There can be no doubt, therefore, that listeriae can enter and multiply in nonphagocytic cells. The intracellular doubling time of listeriae was determined to be 2.5 h. However, this may be an overestimate if, during penetration of MEF by listeriae, some antibiotic gained access to the intracellular confines of MEF and exerted a bacteriostatic effect. Regardless of this possibility, however, progressive *Listeria* proliferation resulted in destruction of the originally infected cells and subsequent infection of other cells. Such an infection process in nonprofessional phagocytes may occur in vivo and contribute to the formation of infectious foci in the liver and spleen during listeriosis. Indeed, a published study of infectious foci in the liver of *Listeria*-infected mice revealed extensive infection of parenchymal cells (18).

It is possible, therefore, that IFN-α/β found in the serum of *Listeria*-infected mice at a time when peak numbers of listeriae are present in the liver and spleen (5, 14, 16, 19) could be produced by parenchymal cells as well as macrophages. Moreover, the IFNs might prove to be functionally important in host resistance. In vitro, interferons have been shown to inhibit the intracellular multiplication of *Shigella* spp. (4), chlamydiae (21), and rickettsiae (13). The possibility that IFN-α/β produced during listeriosis may suppress multiplication of listeriae inside nonprofessional phagocytes deserves investigation. A nonspecific defense mechanism of this type could serve to restrict bacterial growth until an effective cell-mediated immune response can be begun by the host to resolve the infection.

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