

**γ INTERFERON LIMITS ACCESS OF *LISTERIA*
MONOCYTOGENES TO THE MACROPHAGE CYTOPLASM**

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Much of our current understanding of cell-mediated immunity to intracellular pathogens stems from studies on the immune response to the bacterial pathogen *Listeria monocytogenes* (1). It is generally believed that *L. monocytogenes* grows in resident macrophages but is killed by activated or inflammatory macrophages that migrate from the bone marrow to the sites of infection (1).

Although the immune response to *L. monocytogenes* has received a great deal of attention, until recently, little was known about the cell biology of intracellular growth. However, the stages observed during the growth and spread of *L. monocytogenes* in a macrophage cell line have recently been documented (4). It is now clear that *L. monocytogenes* escapes from the host phagosome, exploits the host cytoskeletal machinery, and is presented in a pseudopod-like structure that is apparently recognized and phagocytosed by a neighboring cell. These studies have provided a cell-biological explanation for the absolute requirement for cell-mediated immunity to *L. monocytogenes* infection in mice, i.e., the bacteria never leave the host cytoplasm yet are able to spread cell to cell. It should now be possible to address the precise site(s) at which anti-*L. monocytogenes* immunity is expressed in activated macrophages.

Materials and Methods

Bacterial Strains and Growth Conditions. *L. monocytogenes* strain 10403S and the isogenic hemolysin-negative mutant DP-L215 were the only strains used in this study and were grown as previously described (5).

Cytokines. Murine rIFN- γ was a gift provided by Genentech (South San Francisco, CA) at a concentration of 2.4 mg/ml and a sp act of 1.9×10^7 U/ml. Murine rTNF- α (Genentech) was supplied at a concentration of 0.96 mg/ml and a sp act of 2.9×10^7 U/ml.

Tissue Culture. Primary cultures of resident peritoneal macrophages were established from specific pathogen-free female CD-1, ICR mice (Charles River Breeding Laboratories, Inc., Wilmington, MA), as previously described (6). Resident peritoneal exudate cells (2×10^7) were deposited onto 12-mm round cover slips placed in 60 \times 20-mm petri dishes in 5 ml DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS. After 2 h, nonad-

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herent cells were removed by gently washing with 37°C PBS, pH 7.4, and incubated in the appropriate medium for 40 h.

Fate of L. monocytogenes in Murine Macrophages. *L. monocytogenes* was prepared as previously described (5). Monolayers of peritoneal macrophages grown on cover slips were infected with 2×10^6 bacteria/ml for 30 min. For the 30-min time point, cover slips were washed (by dipping five times in 4°C PBS) and deposited, in triplicate, into 5 ml of sterile distilled water in 15-ml conical tubes. After mixing vigorously for 15 s to lyse the infected cells, dilutions were plated on BHI agar. This time point represents cell-associated bacteria and does not discriminate between intracellular bacteria and those merely attached, although a vast majority of the cell-associated bacteria were intracellular (5, and data not shown). The remaining cover slips were washed three times with 37°C PBS followed by the addition of 5 ml of prewarmed medium. After 30 min, gentamicin sulfate was added to a final concentration of 5 µg/ml. Gentamicin is added to kill any free bacteria that had not entered a macrophage. After 1 h, and at each time point thereafter, the number of intracellular bacteria per cover slip was determined as described above, but without washing in PBS. The data presented in Fig. 1 represent an average of three coverslips. It should be noted that the omission of gentamicin for the first 4 h does not affect the results (5, and data not shown).

EM. Peritoneal macrophages were deposited onto 35 × 10-mm tissue culture petri dishes (Costar, Cambridge, MA) and incubated in the appropriate medium for 40 h. Monolayers of peritoneal macrophages were infected as described above, but with 2×10^7 bacteria/ml. After 4 h, the monolayers were fixed in situ and prepared for EM as previously described (4).

Results and Discussion

Growth of *L. monocytogenes* in resident peritoneal macrophages was not nearly as extensive as was previously seen in a macrophage-like cell line (8). In fact, ~80% of the bacteria associated with the macrophage monolayer after 30 min were killed during the subsequent 90 min, and the survivors only doubled between two and three times (Fig. 1 A). It is not clear why primary peritoneal cells restrict intracellular growth of bacteria, but it may reflect the physiological state of the host cell, as CSF-1-treated or LPS-treated macrophages are more permissive for intracellular growth than untreated cells (data not shown). In contrast to peritoneal macrophages incubated in medium alone, peritoneal macrophages incubated with rIFN-γ completely suppressed any measurable bacterial growth, and continued to exhibit bactericidal activity for the duration of the experiment (Fig. 1 A). This activity was observed with doses of rIFN-γ as low as 1 u/ml (data not shown). Thus, rIFN-γ has a clear and measurable effect on the fate of intracellular *L. monocytogenes*. rTNF had no measurable activity by itself, but in combination with rIFN-γ, there was enhanced bactericidal activity (Fig. 1 A).

It is clear that rINF-γ-treated peritoneal macrophages restrict the growth of intracellular *L. monocytogenes* and continue to exhibit bactericidal activity for the duration of the 8-h experiment. However, it is curious that rINF-γ-treated macrophages exhibit no more bactericidal activity than untreated cells during the first 2 h of infection, a result that was similar to that reported by other investigators (2, 3). Therefore, to directly examine bactericidal activity without error introduced from competition of bacterial growth, we used a hemolysin-negative mutant (*hly*⁻) of *L. monocytogenes*. This mutant is internalized by macrophages to the same degree as the *hly*⁺ strain, but is absolutely defective for intracellular growth (5). Thus, we can directly measure bactericidal activity using this strain. The results clearly showed that there was no difference in the fate of the *hly*⁻ mutant in either untreated or rIFN-γ-treated macrophages (Fig. 1 B). In both cases, the degree of killing resembled that of the

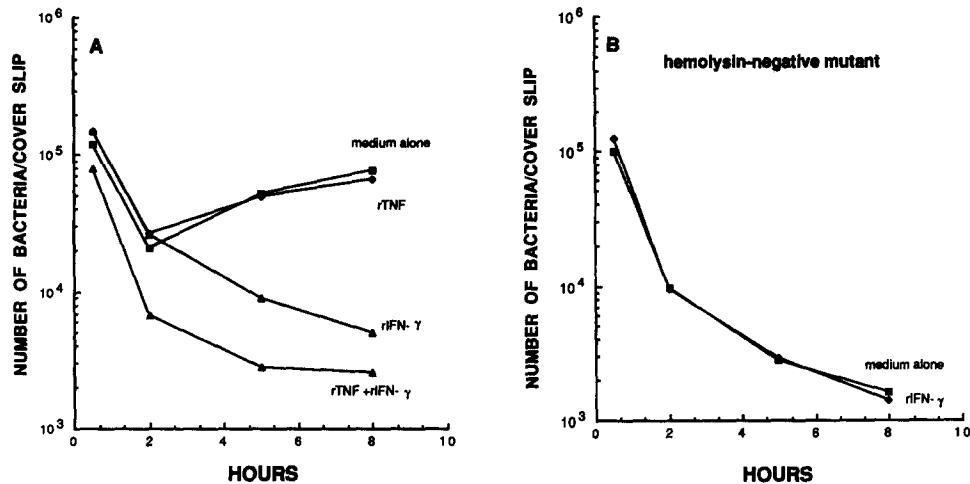


FIGURE 1. (A) Fate of *L. monocytogenes* in mouse peritoneal macrophages treated with cytokines. (Squares) Macrophages incubated with medium alone; (diamonds) macrophages incubated with mouse rTNF- α (1,000 U/ml); (closed triangles) macrophages incubated with rIFN- γ (100 U/ml); and (open triangles) macrophages incubated with rIFN- γ (100 U/ml) plus rTNF- α (1,000 U/ml). (B) Fate of hemolysin-negative *L. monocytogenes* in mouse peritoneal macrophages treated with medium alone or rIFN- γ (100 U/ml).

hly⁺ strain in rIFN- γ -treated cells (Fig. 1 A). Thus, rIFN- γ does not only enhance bactericidal activity, but somehow causes the hly⁺ strain to resemble the hly⁻ strain. Since it is now clear that hly⁺ *L. monocytogenes* grows in the host cytoplasm while the hly⁻ strain is restricted to the endosomal compartment (4, 7), IFN- γ may prevent access of the hly⁺ strain to the cell cytoplasm. Alternatively, rIFN- γ may prevent growth of *L. monocytogenes* in the cytoplasm. To differentiate between these two possibilities, we used EM to examine the intracellular location of the hly⁺ strain in the two macrophage populations.

Monolayers of peritoneal macrophages were infected with *L. monocytogenes* for 4 h, then fixed, sectioned, and examined by EM. Recall, that even in untreated resident macrophages, the majority of the bacteria are killed, and are therefore expected to reside in phagocytic vacuoles. Accordingly, 72% of the bacteria in the untreated macrophage population were found in phagocytic vacuoles (Fig. 2 a and Table I). The other 28% of the bacteria were clearly free in the cytoplasm and half of these were surrounded by actin filaments (Fig. 2, b and c). In a previous study using a macrophage cell line (4), we showed that association with actin filaments is required for cell-to-cell spread of the bacteria. Now, we show that this occurs in primary macrophages as well. Based on our previous work, we hypothesize that bacteria that have associated with actin filaments are probably viable and capable of spreading to an adjacent cell. In the rIFN- γ -treated macrophages, the vast majority of the bacteria were in vacuoles (Fig. 2, d and f), while only 5% were free in the cytoplasm, and none of these were surrounded by actin filaments (Fig. 2 e). Since the three bacteria found in the cytoplasm were free of actin filaments, they were either nonviable, or if viable, probably incapable of cell-to-cell spread. In addition, there were 171 "dots" seen in the rIFN- γ -treated population and none in the untreated population (Fig.

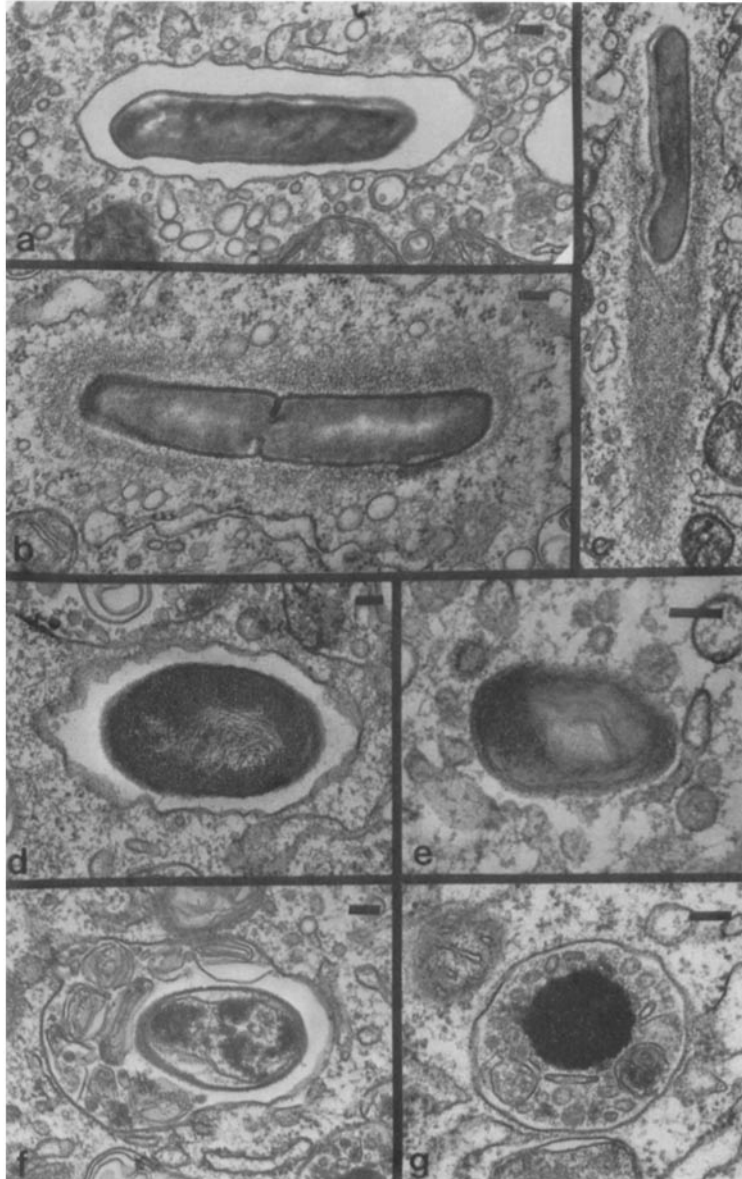


FIGURE 2. Electron micrographs of peritoneal macrophages infected with *L. monocytogenes* for 4 h. (a-c) Macrophages incubated in medium alone; (d-g) macrophages incubated in rIFN- γ (100 U/ml) (bar = 0.1 μ). (a) *L. monocytogenes* present in a phagosome ($\times 33,000$). (b) *L. monocytogenes* in the process of division has entered the cytoplasm and is surrounded by a cloud of actin filaments ($\times 33,000$). (c) *L. monocytogenes* has entered the cytoplasm and has reorganized the actin filaments into an asymmetric tail as a prerequisite to spreading to a neighboring cell ($\times 23,000$). (d) *L. monocytogenes* present in a phagosome of an rIFN- γ -treated cell ($\times 32,000$). (e) A rare instance in which *L. monocytogenes* is found in the cytoplasm of an rIFN- γ -treated macrophage. Notice that the bacteria are not surrounded by actin filaments ($\times 63,000$). (f) *L. monocytogenes* present in a phagosome in which the bacterium appears sick, probably in the early stage of degradation ($\times 33,000$). (g) An electron-dense "dot" that may be the remains of an incompletely digested *L. monocytogenes* ($\times 47,000$).

TABLE I
*Intracellular Location of L. monocytogenes in
 Resident and IFN- γ -treated Peritoneal Macrophages*

| Treatment* | Bacteria [†] | Bacteria in | Bacteria | Bacteria in |
|---------------|-----------------------|-------------|--------------|----------------------------------|
| | | endosome | in cytoplasm | cytoplasm and actin [§] |
| | | % | % | % |
| Medium alone | 50 | 36 (72) | 14 (28) | 7 (14) |
| IFN- γ | 59 | 56 (95) | 3 (5) | 0 |

* Mouse peritoneal macrophages were either treated with 100 U/ml of IFN- γ for 40 h or in medium alone.

[†] The total number of intact bacteria observed by EM in one section.

[§] The dense material surrounding the bacteria was previously identified as F-actin by S₁ decoration (4).

2 g). These dots are electron-dense structures that probably represent digested *L. monocytogenes*, since they were not seen in uninfected rIFN- γ -treated macrophages (data not shown). If so, this would reduce the percentage of bacteria in the cytoplasm to 1.3%, compared with 28% for the control. Another striking difference between the two populations was seen in the morphology of the bacteria. In the untreated population, the majority of the bacteria in vacuoles appeared normal (Fig. 2 a). In contrast, in the rIFN- γ -treated population, the majority of the bacteria appeared "sick" and often showed a separation of the bacterial wall and plasma membrane (Fig. 2 f). Also, there were substantial amounts of membranous material surrounding the bacteria. These data suggest that rIFN- γ -treated macrophages have enhanced ability to digest killed bacteria. This may be partially responsible for enhanced *L. monocytogenes* antigen presentation in IFN- γ -treated macrophages (8).

There are a number of stages in the growth and spread of *L. monocytogenes* in which primary macrophages halt the infection. First, even untreated resident peritoneal macrophages show bactericidal activity and restrict subsequent intracellular growth of *L. monocytogenes* compared with a macrophage cell line or primary fibroblasts (5). In addition, the results of our study are consistent with an effect of IFN- γ in preventing access of the bacteria to the cytoplasmic compartment, thereby enhancing the killing of the bacteria in the phagolysosome. This is a critical step in pathogenicity, since once *L. monocytogenes* enters the cytoplasmic compartment, the infection can spread to adjacent, permissive cells, such as parenchymal cells in the liver and spleen (13).

How might IFN- γ prevent access of *L. monocytogenes* to the cytoplasm? The most obvious mechanism would be to block the action of hemolysin, which is thought to mediate disruption of the phagosomal membrane (4, 7). Since the hemolysin contains a unique, oxygen-labile cysteine (10), it is possible that reactive oxygen intermediates or reactive nitrogen intermediates could render the hemolysin impotent. Interestingly, the production of both reactive oxygen intermediates and reactive nitrogen intermediates is stimulated by IFN- γ and thought to play a role in microbicidal and tumoricidal activity (11).

Summary

The effect of rIFN- γ and rTNF on the fate of hemolytic and nonhemolytic (hly⁻) *Listeria monocytogenes* in cultured mouse peritoneal macrophages was investigated. In

untreated macrophages, ~80% of the hemolytic bacteria were killed during the first 2 h of incubation, but the survivors doubled between two and three times. In rIFN- γ -treated macrophages, although the bacterial killing was identical to the controls during the first 2 h, there was no subsequent bacterial growth, and bactericidal activity continued for the duration of the experiment. rTNF has no effect by itself, but acted synergistically with rIFN- γ to promote bacterial killing. Infected macrophages with or without rIFN- γ were examined by EM. The results clearly showed that the role of rIFN- γ was to prevent access of *L. monocytogenes* to the macrophage cytoplasm, which would prevent cell-to-cell spread of the bacteria. In addition, rIFN- γ -treated macrophages exhibited enhanced digestive capacity of the intracellular bacteria.

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