Immunology 1992 **77** 354–361

Intravenous injection of interferon-γ inhibits the proliferation of *Listeria monocytogenes* in the liver but not in the spleen and peritoneal cavity

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Accepted for publication 25 June 1992

SUMMARY

In the present study the effects of intravenous administration of recombinant interferon-γ (IFN-γ) on both the proliferation of *Listeria monocytogenes* in the liver and spleen of mice and the listericidal activity of their peritoneal macrophages were investigated. A single intravenous injection of 1×10^6 U or three injections of 2×10^5 U recombinant IFN-γ (rIFN-γ) induced optimal activation of resident and exudate peritoneal macrophages, as judged by their ability to inhibit the intracellular proliferation of *Toxoplasma gondii* and their enhanced release of H_2O_2 and NO_2^-. The rate of intracellular killing of *L. monocytogenes* by the rIFN-γ-activated resident and exudate macrophages was not higher than that by resident macrophages. Addition of 10 ng lipopolysaccharides (LPS) to the rIFN-γ also did not enhance the bactericidal activity of the activated peritoneal macrophages. The decrease in the number of *L. monocytogenes* in the peritoneal cavity of mice that had received an i.p. injection of 1×10^4 U rIFN-γ was similar to that in control mice. Intravenous administration of 1×10^5 rIFN-γ activated cells in the liver, as indicated by the increased expression of Ia antigen, and reduced the rate of proliferation of *L. monocytogenes* in the liver relative to that in control mice when 0·1 LD_{50} or 1 LD_{50} *L. monocytogenes* were injected. However, when 10 LD_{50} *L. monocytogenes* were administered there was no effect on their proliferation. The number of *L. monocytogenes* found initially in the spleen of rIFN-γ-treated mice was 20–30% of that in the spleen of control mice, but the rate of proliferation of *L. monocytogenes* was not reduced. These divergent results for the proliferation of *L. monocytogenes* in the liver, spleen and peritoneal cavity indicate that cells other than macrophages and/or as yet unknown local factors play an important role in the listericidal activity.

INTRODUCTION

Activated macrophages are considered to be the primary effector cells in the host defence against infections with intracellular pathogens such as *Toxoplasma gondii*, *Listeria monocytogenes* and Mycobacteria spp.[1] Peritoneal macrophages activated during an infection of mice with bacillus Calmette–Guérin (BCG) followed by an i.p. injection of mycobacterial antigen (PPD) or with *L. monocytogenes* kill *L. monocytogenes* faster than normal resident macrophages.[2,3] Macrophages become activated in response to cytokines and there is abundant evidence that interferon-γ (IFN-γ) is the main cytokine involved.[4,5] Since the identification of IFN-γ as a macrophage-activating factor, much effort has gone into identification of its *in vivo* and *in vitro* effects on the immune response. Most of the studies that focused on the activation of macrophages by this cytokine were performed *in vitro*[6,7] after injection, e.g. into the peritoneal cavity,[7] or by injecting neutralizing antibodies against IFN-γ.[4] Murine macrophages activated by recombinant IFN-γ (rIFN-γ) *in vivo* or *in vitro* inhibit the intracellular proliferation of various protozoa, display an enhanced ability to secrete reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) and become tumoricidal after a second stimulus, for example endotoxin.[8–14] Nevertheless, the importance of the role played by IFN-γ in the resistance to infections with various facultative intracellular bacteria is still not clear and the findings are contradictory. It has been reported that intravenous (i.v.) injection of rIFN-γ decreases the number of *L. monocytogenes* in the spleen and liver of mice,[15,16] but in a previous study we found no effect of rIFN-γ on the proliferation of *L. monocytogenes* in these organs.[7] Moreover, macrophages activated *in vitro* with rIFN-γ do not exhibit enhanced listericidal activity[6] and similar results were found after an i.p. injection

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of rIFN-γ.[7] It is possible that these contradictory results are due to differences in the dose of injected rIFN-γ or the number of bacteria used to infect the mice. During an infection of mice with *L. monocytogenes* bacteria proliferate in the liver and spleen and IFN-γ enters the circulation.[17] Circulating IFN-γ is probably involved in the activation of macrophages at distant sites, e.g. in the peritoneal cavity. These considerations prompted us to investigate the effects of i.v. injections of rIFN-γ on the proliferation of *L. monocytogenes* in the liver, spleen and peritoneal cavity of mice, on the activation of macrophages in the liver and peritoneal cavity, and on the listericidal activity of peritoneal macrophages. The aim of the study was to compare the listericidal activity of activated macrophages *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

Specific pathogen-free female CBA/J mice weighing 20–30 g were purchased from IFFA Credo (Saint Germaine-sur-L'Abersle, France). Sterilized food and tap water were given *ad libitum*.

Micro-organisms

Virulent *L. monocytogenes* (strain EGD) [LD_{50} 5×10^3 colony-forming units (CFU) for CBA/J mice] were stored in tryptose phosphate broth (TPB) (Oxoid Ltd, Basingstoke, U.K.) supplemented with 10% (v/v) DMSO at −70°. Samples of the frozen stock were cultured for 18 hr at 37° in TPB. Bacteria were collected by centrifugation (10 min, 1500 ***g***), washed twice with phosphate-buffered saline (pH 7·4) (PBS) and suspended at appropriate bacterial concentrations in Hanks' balanced salt solution supplemented with 0·1% gelatin (gelatin–HBSS) or in saline. The virulence of *L. monocytogenes* was maintained by repeated passage through mice.

The virulent RH strain of *Toxoplasma gondii* was maintained by biweekly intraperitoneal (i.p.) passage through CBA mice. Two or three days after i.p. injection, the protozoa were collected by peritoneal lavage, as described previously.[3] The toxoplasma were then counted in a haemocytometer and suspended at a concentration of 1×10^6/ml in RPMI-1640 medium (Flow Laboratories, Rockville, MD) containing 10% heat-inactivated (30 min, 56°) foetal bovine serum (Flow Laboratories, Irvine, U.K.), 100 U/ml penicillin, and 50 μg/ml streptomycin, hereafter referred to as medium.

Macrophages

Peritoneal macrophages were harvested with 2 ml ice-cold PBS containing 50 U/ml heparin, as described previously.[18] In all experiments resident peritoneal macrophages were used unless stated otherwise. Exudate peritoneal macrophages were obtained from mice injected i.p. with 10% proteose pepton 3 days before cell harvesting. This composition of the cell suspension was determined by analysis of cytospin preparations. The peroxidase reactivity of the cells was determined as described previously.[19] Suspensions of cells from normal mice contained less than 5% peroxidase-positive macrophages and from mice injected i.p. with proteose pepton approximately 20% peroxidase-positive macrophages; i.v. injection of rIFN-γ did not change these percentages. All suspensions used contained less than 5% granulocytes.

Interferon-γ

Recombinant rat IFN-γ (rIFN-γ) was produced in Chinese hamster ovary (CHO) cells. The cytokine was stored in lyophilized form at 4° and diluted in pyrogen-free saline immediately before i.v. or i.p. injection. All reagents contained less than 0·08 ng LPS/ml, as determined with the Limulus lysate assay. In some experiments phenol-extracted lipopolysaccharide (LPS) from *S. typhimurium* (Sigma, St Louis, MO) was added to rIFN-γ.

Assessment of macrophage activation

Intracellular proliferation of Toxoplasma gondii. Peritoneal macrophages were infected with *T. gondii* as described previously.[3] In short, macrophages were suspended in medium at a concentration of 1×10^6/ml, and 2 ml were plated onto 35-mm plastic culture dishes (Falcon, Lincoln Park, NJ) containing three 12-mm round glass coverslips. After 2 hr at 37° in 7·5% CO_2, non-adherent cells were removed by washing. After addition of 1–2 ml medium containing 1×10^6 toxoplasma/ml, the cells were incubated for 30 min at 37° in 7·5% CO_2. After washing to remove non-ingested toxoplasmas, the cells on one coverslip were fixed and then stained with Giemsa for determination of the percentage infected macrophages at 0 hr. The experimental conditions were chosen such that 35% of the macrophages were infected. Fresh medium was added to the other coverslips which were incubated for another 18 hr at 37° in 7·5% CO_2 before fixation and staining with Giemsa; the number of toxoplasma/100 macrophages was then determined microscopically. If the percentage infected macrophages at 18 hr had increased by more than 7%, the results were discarded. The results of the assay were expressed as the fold increase, i.e. the ratio of the number of toxoplasma/100 macrophages after 18 hr of incubation to the number of toxoplasma/100 macrophages at 0 hr.

Measurement of H_2O_2 and NO_2^- release by peritoneal macrophages. The release of hydrogen peroxide by macrophages during stimulation with 100 ng/ml phorbol myristate acetate (PMA; Consolidated Midland, Brewster, NY) was measured according to the horseradish peroxidase-mediated H_2O_2-dependent oxidation of homovanillic acid.[20]

The amount of NO_2^- present in the culture supernatant after an incubation period for macrophages of 24 hr *in vitro* served as a measure for the amount of generated RNI by the cells and was determine using Griess reagent.[13]

Intracellular killing of Listeria monocytogenes

The intracellular killing of bacteria was assessed after *in vitro* phagocytosis of preopsonized *L. monocytogenes*, as previously described.[3] A suspension containing 5×10^6 macrophages and 5×10^6 preopsonized bacteria/ml was incubated for 20 min at 37° and 4 rpm. After phagocytosis, the suspension was centrifuged at 110 ***g*** for 4 min and the cells were washed three times with ice-cold gelatin–HBSS to remove extracellular bacteria. Macrophages containing bacteria were then resuspended at the original concentration in gelatin–HBSS containing 10% (v/v) inactivated rabbit antiserum. At various intervals, 50-μl samples were taken and the macrophages lysed by addition to 450 μl water containing 0·01% (v/v) bovine serum albumin (BSA) under vigorous mixing. Serial 10-fold dilutions were plated onto blood-agar plates and the number of viable bacteria was determined microbiologically. Colony counts were used to

calculate the number of bacteria. At the end of the assay the viability of the macrophages, as indicated by trypan-blue exclusion, exceeded 90%.

The rate constant for intracellular killing (K_k), which is a measure of the intracellular killing of bacteria, was calculated according to the equation: $K_k = [\ln N(t=0) - \ln N(t)]/t$, in which $N(t)$ is the number of viable bacteria at time t (min) and $N(t=0)$ the number of viable bacteria at the beginning of the assay.[2]

In vivo *killing of* Listeria monocytogenes *in the peritoneal cavity*
Mice received an i.p. injection of 1×10^4 U rIFN-γ 18 hr before an i.p. injection of 1×10^7 *L. monocytogenes*. To determine the decrease in the total number of *L. monocytogenes*, i.e. both intracellular and extracellular bacteria, the mice were killed and the peritoneal cells were harvested under standard conditions using exactly 2 ml cold PBS with 50 U heparin. A 500-μl sample of this peritoneal fluid was added to 4·5 ml water containing 0·01% (w/v) BSA; after vigorous mixing the number of micro-organisms was determined microbiologically.

Proliferation of Listeria monocytogenes *in liver and spleen*
Mice received an i.v. injection of rIFN-γ 18 hr before an i.v. injection of *L. monocytogenes*. At various times during the infection several animals were killed and the liver and spleen were isolated and homogenized in 2 ml saline in a tissue homogenizer (type X-1020; Ystral GmbH, Döttingen, Germany). Serial 10-fold dilutions of the organ suspensions were plated onto blood–agar plates and colonies were counted after 18–24 hr of incubation at 37°. Colony counts yielded the CFU of *L. monocytogenes*/organ.

Detection of Ia antigen in the liver
Mice received a single i.v. injection of 1×10^5 U rIFN-γ 18 hr before an i.v. injection of *L. monocytogenes*. At various times during the infection mice were killed, the livers were removed and snap-frozen in melting isopentane (Merck, Darmstadt, Germany) in a plastic-capped syringe in liquid nitrogen, and 5-μm thick cryostat sections were cut and fixed in 0·5% glutaraldehyde. After rinsing with saline Ia antigen expression was determined using the monoclonal antibody ERTR-1 (a gift from Dr P. J. M. Leenen, Dept. of Cell Biology, Erasmus University, Rotterdam, The Netherlands,[21] as described previously.[22] The number of Ia antigen positive cells/mm^2 tissue section was determined microscopically.

Analysis of data
Results are expressed as the mean ± SD of at least three independent experiments unless otherwise indicated. The Mann–Whitney U-test and Kruskall–Wallis analysis were used to compare the characteristics of activation and the rate constants of intracellular killing. Comparison of the proliferation of bacteria in the organs was achieved with multiple regression analysis. For all analyses the level of significance was set at 0·05.

RESULTS

Assessment of the optimal dose of i.v. injected rIFN-γ

The proliferation of *T. gondii* in peritoneal macrophages harvested 24 hr after i.v. injection of 1×10^6 U rIFN-γ was significantly ($P < 0{\cdot}01$) less than in resident macrophages (Table 1). Maximum inhibition of protozoal proliferation by the macrophages was observed 24 hr after injection of rIFN-γ; the macrophages subsequently became deactivated, complete deactivation occurred after 36 hr. Injection of mice with a lower dose of rIFN-γ, i.e. 2×10^5 rIFN-γ alone or together with 10 ng LPS, did not affect the *in vitro* intracellular proliferation of *T. gondii* in the macrophages (Table 1).

Twenty-four hours after an i.v. injection of 1×10^6 U rIFN-γ, peritoneal macrophages showed a fourfold increase in the release of H_2O_2 relative to the amount released by resident macrophages (Table 1); a fourfold increase in H_2O_2 release was also found for rIFN-γ-activated exudate peritoneal macrophages (data not shown). Macrophages from mice that received a single i.v. injection of 2×10^5 U rIFN-γ alone or together with 10 ng LPS did not release more H_2O_2 than resident macrophages (Table 1).

Since rIFN-γ is cleared from the circulation rapidly,[23] we investigated whether repeated injections of rIFN-γ would give better activation of peritoneal macrophages, which in turn could lead to enhanced listericidal activity (see below). Mice that

Table 1. Effect of a single i.v. injection of rIFN-γ on the intracellular proliferation of *T. gondii*, the release of H_2O_2, and the rate constant for intracellular killing of *L. monocytogenes* by murine peritoneal macrophages†

Treatment	Intracellular proliferation of *T. gondii*‡ (fold increase)	H_2O_2 release§ (nmol)	Rate constant for intracellular killing of *L. monocytogenes in vitro*¶ (K_k/min)
Saline	6·6 ± 1·4	0·40 ± 0·01	0·036 ± 0·007
1×10^6 U rIFN-γ	1·7 ± 0·9*	1·90 ± 0·65*	0·039 ± 0·015
2×10^5 U rIFN-γ	7·8 ± 0·5	0·50 ± 0·20	ND
2×10^5 U rIFN-γ plus 10 ng LPS	5·7 ± 1·1	0·50 ± 0·09	ND

† Mice received an i.v. injection of rIFN-γ 24 hr before harvesting of the peritoneal macrophages.

‡ Values are the mean ± SD of four experiments as the fold increase in the number of *T. gondii*/100 macrophages after an 18-hr infection period.

§ Values are the mean ± SD of four experiments and are expressed as nmol H_2O_2 released by 1×10^6 macrophages during 1 hr.

¶ Values are the mean ± SD of rate constants for intracellular killing of *L. monocytogenes* determined at 15-min intervals during the first 30 min of intracellular killing ($n = 5$).

* Value is significantly ($P < 0{\cdot}05$) different relative to the respective value for the saline-treated mice.

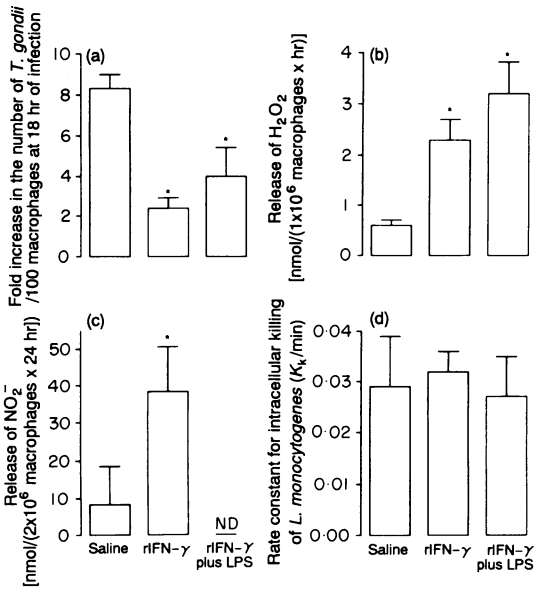

Figure 1. Characteristics of mouse peritoneal macrophages from rIFN-γ-treated and control mice. Mice received three consecutive i.v. injections of 2×10^5 U rIFN-γ alone or together with 10 ng LPS 24, 21 and 18 hr before harvesting of the peritoneal macrophages; control mice received saline. The intracellular proliferation of *T. gondii* (a), the release of H_2O_2 (b) and NO_2^- (c), and the rate constant for intracellular killing of *L. monocytogenes* (d) by these macrophages was assessed. Each bar represents the mean ± SD of at least three experiments. ND, not done. $^*P < 0.05$.

received repeated doses of 1×10^6 U rIFN-γ died soon after the second injection. Lower successive doses, i.e. 2×10^5 U rIFN-γ given i.v. 24, 21 and 18 hr before harvesting activated the peritoneal macrophages to a similar degree as a single i.v. injection of 1×10^6 U rIFN-γ as judged by their ability to inhibit the intracellular proliferation of *T. gondii* (Fig. 1a, Table 1). Three successive i.v. injections of 2×10^5 U rIFN-γ and 10 ng of LPS also resulted in inhibition of the intracellular proliferation of *T. gondii* in the macrophages, but the effect was slightly less than after three injections of only rIFN-γ (Fig. 1a).

The release of H_2O_2 by macrophages from mice that received three successive i.v. injections of rIFN-γ was significantly ($P < 0.01$) higher than that by macrophages from control mice (Fig. 1b) and similar to that found after a single i.v. injection of 1×10^6 U rIFN-γ (Table 1). Addition of 10 ng LPS to rIFN-γ resulted in a small but significant ($P < 0.05$) increase in the release of H_2O_2 relative to three injections of only rIFN-γ (Fig. 1b). Three i.v. injections of 10 ng LPS alone did not activate the macrophages either to inhibit the intracellular proliferation of *T. gondii* or to release more H_2O_2 (data not shown).

The release of NO_2^- by macrophages from mice that received three i.v. injections of rIFN-γ was significantly ($P < 0.05$) enhanced relative to that by macrophages from control mice (Fig. 1c).

Proliferation of *Listeria monocytogenes* in the liver and spleen of rIFN-γ-treated and normal mice

The rate constant for the proliferation of *L. monocytogenes* in the liver and spleen of mice that received a single injection of 1×10^6 U rIFN-γ followed by 10 LD_{50} of the bacteria was similar to that found for control mice during the first 48 hr of infection (data not shown); the same was also found after two i.v. injections of 5×10^4 U rIFN-γ.[7] Similar results were found after an i.v. injection of 1×10^5 U rIFN-γ and 10 LD_{50} *L. monocytogenes* (Fig. 2a, Table 2); both rIFN-γ-treated and control mice died around Day 3 of infection. The rate constants for the proliferation of *L. monocytogenes* in the liver of mice that received an i.v. injection of 1×10^5 U rIFN-γ and 0·1 LD_{50} or 1 LD_{50} *L. monocytogenes* were significantly ($P < 0.05$) lower than the rate constants for bacterial proliferation in the liver of control mice (Fig. 2b, c, Table 2).

Twenty-four hours after injection of 0·1 or 1 LD_{50} *L. monocytogenes* the number of bacteria recovered from the spleen of rIFN-γ-treated mice was 20–30% of that found in the spleen of control mice (Fig. 2b, c), but the rate constants for the proliferation of *L. monocytogenes* in the spleen of rIFN-γ-treated and control mice from Days 1 to 3 of infection were similar (Table 2).

In another experiment, mice received an i.v. injection of 1×10^5 U rIFN-γ 18 hr before i.v. injection of two LD_{50} *L. monocytogenes*. On Day 7 of infection all control mice that had not received rIFN-γ died, whereas all mice that had received rIFN-γ were still alive at the last day of the experiment, i.e. Day 12 of infection.

Expression of Ia antigen in the liver

On Days 1, 2 and 5 of infection with *L. monocytogenes* significantly ($P < 0.05$) more cells that exhibited marked expression of Ia antigen were present in the livers of rIFN-γ-treated mice than in control mice (Fig. 3). Moreover, during the infection more inflammatory foci containing cells that exhibited marked expression of Ia antigen developed in the liver of rIFN-γ-treated mice than in the liver of control mice (data not shown).

Intracellular killing of *Listeria monocytogenes* by rIFN-γ-activated peritoneal macrophages

To investigate whether the decrease in the proliferation of *L. monocytogenes* in the livers of rIFN-γ-treated mice could be due to enhanced intracellular killing of the bacteria by activated macrophages in this organ, we investigated the *in vitro* intracellular killing of *L. monocytogenes* by peritoneal macrophages activated by a single i.v. injection of 1×10^6 U rIFN-γ or three injections of 2×10^5 U rIFN-γ. After *in vitro* phagocytosis at a bacteria-to-cell ratio of 1 : 1, the number of viable *L. monocytogenes* in macrophages from mice collected after a single i.v. injection of 1×10^6 U rIFN-γ [$1{\cdot}5(\pm 0{\cdot}72) \times 10^6/5 \times 10^6$ macrophages] was similar to that in macrophages from control mice [$1{\cdot}7(\pm 0{\cdot}84) \times 10^6/5 \times 10^6$ macrophages].

In rIFN-γ-activated macrophages and macrophages from control mice the course of intracellular killing was biphasic: the number of *L. monocytogenes* decreased linearly during the first 30 min and remained constant or increased slightly during the next 30 min (data not shown). During both intervals the changes in the numbers of viable intracellular *L. monocytogenes* in macrophages from rIFN-γ-treated and control mice were comparable ($P > 0.1$) with similar rate constants for intracellular killing (Table 1). The rate constants for the intracellular killing of *L. monocytogenes* by rIFN-γ-activated exudate macrophages (K_k/min = 0·046) and control exudate macrophages (K_k/min = 0·050) were similar ($P > 0.1$) as well.

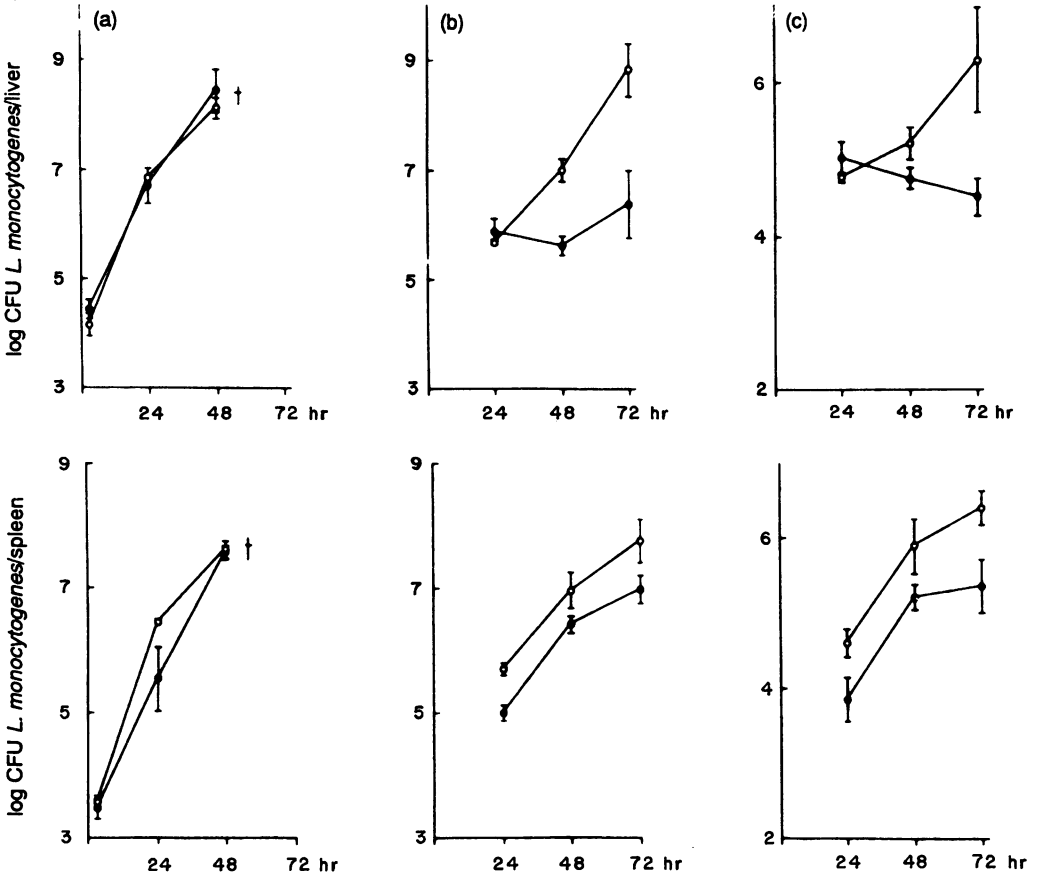

Figure 2. Course of the number of viable *L. monocytogenes* in the liver (upper panel) and the spleen (lower panel) of rIFN-γ-treated (●) and control (○) mice during the first 3 days of infection. The treated mice received 1×10^5 U rIFN-γ i.v. and the controls received saline i.v. Eighteen hours later all animals were injected with 5×10^4 viable *L. monocytogenes* (a), 5×10^3 viable *L. monocytogenes* (b) or 5×10^2 viable *L. monocytogenes* (c) i.v. Values are the means ± SD for four to five mice.

Table 2. Rate constant for proliferation of *L. monocytogenes* in the liver and spleen of control and rIFN-γ-treated mice†

Organ	Injected bacteria/ mouse	LD_{50}	Rate constant for proliferation‡	
			Control mice (/hr)	rIFN-γ-treated mice (/hr)
Liver	5×10^4	10	0·087 ± 0·006	0·088 ± 0·010
	5×10^3	1	0·064 ± 0·011	0·016 ± 0·003*
	5×10^2	0·1	0·032 ± 0·014	−0·010 ± 0·007§
Spleen	5×10^4	10	0·089 ± 0·003	0·087 ± 0·008
	5×10^3	1	0·041 ± 0·006	0·041 ± 0·008
	5×10^2	0·1	0·036 ± 0·008	0·033 ± 0·006

† Mice received a single i.v. injection of 1×10^5 U rIFN-γ or saline and 18 hr later an i.v. injection of *L. monocytogenes*.

‡ The numbers of bacteria in the liver and spleen were determined 3, 24 and 48 hr after i.v. injection of 10 LD_{50} *L. monocytogenes*, and 24, 48 and 72 hr after i.v. injection of 1 LD_{50} and 0·1 LD_{50} *L. monocytogenes*. At each time-point the liver and spleen of four to five mice were investigated; values represent the mean ± SD.

* Rate constant for proliferation is significantly ($P < 0{\cdot}01$) decreased relative to that for control mice.

§ Negative value indicates a decrease in the number of bacteria.

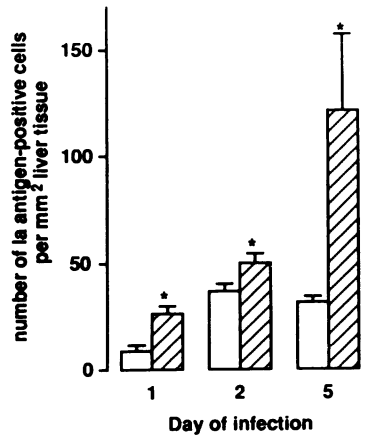

Figure 3. Number of cells markedly expressing Ia antigen in the livers of rIFN-γ-treated (■) and control (□) mice infected with *L. monocytogenes*. Mice received an i.v. injection of 1×10^5 U rIFN-γ 18 hr before an i.v. injection of 1 LD_{50} *L. monocytogenes*. The number of cells markedly expressing Ia antigen/mm² liver tissue was assessed on Days 1, 2 and 5 of infection and the values are the means ± SEM for three mice.

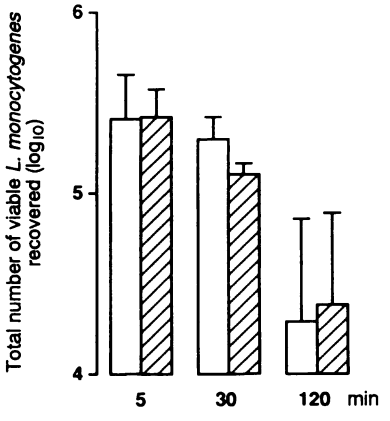

Figure 4. Total number of *L. monocytogenes* in the peritoneal cavity of rIFN-γ-treated (■) and control mice (□). Mice received an i.p. injection of 1×10^4 U rIFN-γ or saline and 24 hr later an i.p. injection of 1×10^7 viable *L. monocytogenes*. The total number of bacteria, i.e. ingested and non-ingested, in the peritoneal cavity was determined 5, 30 and 120 min after injection of the bacteria. Each bar represents the mean ± SD for four mice.

When the mice received three successive i.v. injections of 2×10^5 U rIFN-γ alone or together with 10 ng LPS, the rate constants for the intracellular killing of *L. monocytogenes* for rIFN-γ-activated and control macrophages were similar as well (Fig. 1d). Intravenous injection of 10 ng LPS alone had no effect on the rate constants for intracellular killing (data not shown). During continuous phagocytosis,[24] rIFN-γ-activated macrophages also did not kill *L. monocytogenes* faster than control macrophages when the bacteria-to-cell ratios were 10:1, 1:1 and 1:10 (data not shown).

To investigate the role of rIFN-γ-activated macrophages in the killing of *L. monocytogenes in vivo*, mice received an i.p. injection of the bacteria 18 hr after an i.p. injection of 1×10^4 U rIFN-γ, at which time the peritoneal macrophages were activated as judged by their toxoplasmastatic activity and enhanced release of H_2O_2 (data not shown).[17] The total numbers of viable *L. monocytogenes*, i.e. ingested and non-ingested bacteria, recovered 5, 30 and 120 min after an i.p. injection of the bacteria were almost similar ($P > 0.05$) for rIFN-γ-treated mice and control mice (Fig. 4). Thirty minutes after injection of the bacteria 8·4 ± 4·0% of the cell population from the peritoneal cavity consisted of peroxidase-positive cells, i.e, exudate macrophages and granulocytes; at 120 min the majority of the cells in the peritoneal cavity (61·7 ± 9·1%) was peroxidase-positive, being mainly granulocytes. The cell composition in the peritoneal cavity of rIFN-γ-treated was similar to that in control mice at all time-points.

DISCUSSION

New observations of this study are that i.v. administration of high doses of rIFN-γ leads to inhibition of the proliferation of *L. monocytogenes* in the liver but not the spleen of infected mice and that this effect is dependent on the number of injected bacteria. Furthermore, our results demonstrate that this inhibition of bacterial proliferation is most likely not due to enhanced intracellular killing by rIFN-γ-activated macrophages in the liver since activated peritoneal macrophages from rIFN-γ-treated mice do not exhibit enhanced listericidal activity *in vivo* and *in vitro*.

Earlier studies on the effect of rIFN-γ administration on infections with *L. monocytogenes* focused on proliferation of this organism in the spleen[16] or the number of bacteria recovered from the organs at one specific time-point during the infection, e.g. on Day 2.[15] In the present study the effect of i.v. rIFN-γ on the proliferation of *L. monocytogenes* in the liver and spleen during a 72-hr period was studied. After injection of 0·1 LD_{50} or 1 LD_{50} *L. monocytogenes* the rate of proliferation of the bacteria in the liver of rIFN-γ-treated mice was lower than that in control mice; however rIFN-γ alone is not sufficient to protect mice against infections with very high numbers of *L. monocytogenes*, since proliferation of the bacteria in the liver of mice that were given 10 LD_{50} *L. monocytogenes* was the same in rIFN-γ-treated and control animals, and in both groups all mice died. The proliferation of *L. monocytogenes* in the spleen of rIFN-γ-treated mice was not reduced, irrespective of the number of bacteria injected, although the number of *L. monocytogenes* recovered at the start of the experiment from the spleen of rIFN-γ-treated mice that received 0·1 LD_{50} or 1 LD_{50} *L. monocytogenes* was 20–30% of that found in the spleen of control mice. As yet, we cannot explain why rIFN-γ affects the proliferation of *L. monocytogenes* in the liver but not in the spleen. It might be that these divergent effects of rIFN-γ are due to differences in the cell composition of the organs.

Activation of resident or exudate peritoneal macrophages was demonstrated by their ability to inhibit the intracellular proliferation of *T. gondii* and release enhanced amounts of ROI and RNI, as measured by the enhanced amounts of H_2O_2 and NO_2^- respectively, relative to normal resident or exudate macrophages. Despite the release of these microbicidal agents by the activated peritoneal macrophages the listericidal activity of these macrophages *in vitro* and *in vivo* was not enhanced. It is possible that activated peritoneal macrophages are not representative of a study on the enhanced bactericidal activity of

activated macrophages in organs such as the liver or spleen. However, in earlier studies we demonstrated that inhibition of the proliferation of *L. monocytogenes* in the liver of *L. monocytogenes*-activated and BCG-activated mice correlates with an enhanced listericidal activity of their peritoneal macrophages.[2,3] Another possibility is that the duration of the intracellular killing assay, i.e. 60 min, was not long enough to allow observation of the differences between rIFN-γ-activated and resident macrophages. However, during the first 1–3 hr of the assay no differences in the intracellular killing of *L. monocytogenes* by rIFN-γ-activated and control macrophages was observed.[7,25,26] After longer incubation periods, surface-adherent macrophages inhibited the intracellular proliferation of *L. monocytogenes* whereas the bacteria proliferated in control macrophages.[26] It is not known, however, whether effects on the bactericidal activity of isolated macrophages observed in *in vitro* studies lasting longer than a few hours are representative of the *in vivo* situation. Together, these results indicate that the intracellular killing of *L. monocytogenes* by macrophages consists of two phases. During the initial phase the rate for intracellular killing of the bacteria is similar for rIFN-γ-activated and control macrophages; during the second phase the intracellular proliferation of the bacteria is inhibited in rIFN-γ-activated macrophages but not in control macrophages.

The contradictory findings on the effect of rIFN-γ on the proliferation of *L. monocytogenes* in the liver and on the listericidal activity of activated peritoneal macrophages are difficult to explain. *In vivo*, during an inflammatory response, an influx of granulocytes and monocytes might contribute to the elimination of infecting micro-organisms from the liver.[27,28] However morphological studies did not reveal increased numbers of granulocytes and exudate macrophages, i.e. monocyte-derived cells, in the liver of rIFN-γ-treated *L. monocytogenes*-infected mice relative to that in control mice infected with *L. monocytogenes* (J. A. M. Langermans, M. E. B. van der Hulst, P. H. Nibbering and R. van Furth in preparation). Moreover, at a time that granulocytes dominated during a *Listeria* infection of the peritoneal cavity no difference in the decrease in number of bacteria was found between rIFN-γ-treated and control mice. During the *Listeria* infection, more cells exhibited marked expression of Ia antigen in the liver of rIFN-γ-treated mice than in control mice. After injection of rIFN-γ Ia-antigen expression by peritoneal macrophages is also increased.[13] Since in the liver and peritoneal cavity of rIFN-γ-treated mice activation of macrophages occurred, the difference in elimination of *L. monocytogenes* from the two sites cannot be explained by a difference in macrophage activation. Probably other types of cell are involved in the elimination of *L. monocytogenes* from the liver.

The current view on the pathogenesis of *L. monocytogenes* infections of the liver might explain the divergent courses of the number of bacteria in control and rIFN-γ-treated mice. In the liver of control mice *L. monocytogenes* are first ingested by macrophages and become localized in phagolysosomes; from there the bacteria escape into the cytoplasm of these cells.[26] Next, *L. monocytogenes* spread from cell to cell, invade the hepatocytes and multiply, resulting in an increase in the number of bacteria in the liver.[26,29,30] During this period granulocytes migrate to the site of inflammation and lyse infected hepatocytes, thus inducing necrosis and release of the intracellular bacteria.[28] Next, phagocytes migrate to these necrotic lesions and ingest and kill the bacteria, which leads to inhibition of the proliferation of *L. monocytogenes* in the liver.[27,28] The number of bacteria decreases after full development of the cellular immunity. In rIFN-γ-treated mice *L. monocytogenes* are also ingested by macrophages in the liver, but activation of these cells by rIFN-γ-inhibits the escape of *L. monocytogenes* from the phagolysosomes,[26] although a number of bacteria might still invade the hepatocytes. Thus, the bacteria in the macrophages remain exposed to their bactericidal action, which leads to a decrease in the total number of *L. monocytogenes* in the liver. The mechanisms involved in the killing of *L. monocytogenes* by the macrophages are not clear since the enhanced generation of ROI and RNI by these cells did not result in increased listericidal activity. It may be that the killing of *L. monocytogenes* by macrophages is mediated by oxygen-independent microbicidal mechanisms. In addition, rIFN-γ-activated Kupffer cells release factors that activate hepatocytes to generate RNI.[31] Such activated hepatocytes have been demonstrated to inhibit the intracellular proliferation of *Plasmodium berghei*.[32] It is feasible that comparable mechanisms inhibit the proliferation of *L. monocytogenes* in these activated hepatocytes. Recently, it has been demonstrated that after challenge of *Listeria*-immunized mice with a lethal number of *L. monocytogenes* and subsequent isolation of the liver cells after various intervals, the bacteria are eliminated faster from the hepatocytes of immune mice than of non-immune mice.[33] Thus, an indirect effect of stimulation with rIFN-γ is the activation of hepatocytes by factors released by activated macrophages. However, when the number of *L. monocytogenes* injected into rIFN-γ-treated mice is very large, both the activated macrophages and hepatocytes cannot cope with the bacteria and the number of *L. monocytogenes* in the liver increases.

REFERENCES

1. Hahn H. & Kaufmann S.H.E. (1983) The role of cell-mediated immunity in bacterial infections. *Rev infect. Dis.* **3,** 1221.
2. van Dissel J.T., Stikkelbroeck J.J.M., van den Barselaar M.Th., Sluiter W., Leijh P.C.J. & van Furth R. (1987) Divergent changes in antimicrobial activity after immunologic activation of mouse peritoneal macrophages. *J. Immunol.* **139,** 1665.
3. Langermans J.A.M., van der Hulst M.E.B., Nibbering P.H. & van Furth R. (1990) Activation of mouse peritoneal macrophages during infection with *Salmonella typhimurium* does not result in enhanced intracellular killing. *J. Immunol.* **144,** 4340.
4. Buchmeier N.A. & Schreiber R.D. (1985) Requirement of endogenous interferon-γ production for resolution of *Listeria monocytogenes* infection. *Proc. natl. Acad. Sci. U.S.A.* **82,** 7407.
5. Murray H.W. (1988) Interferon-gamma, the activated macrophage, and host defense against microbial challenge. *Ann. intern. Med.* **108,** 595.
6. Campbell P.A., Canone B.P. & Cook J.L. (1988) Mouse macrophages stimulated by recombinant gamma interferon to kill tumor cells are not bactericidal for the facultative intracellular bacterium *Listeria monocytogenes*. *Infect. Immun.* **56,** 1371.
7. van Dissel J.T., Stikkelbroeck J.J.M., Michel B.C., van den Barselaar M.Th., Leijh P.C.J. & van Furth R. (1987) Inability of recombinant interferon-γ to activate the antibacterial activity of mouse peritoneal macrophages against *Listeria monocytogenes* and *Salmonella typhimurium*. *J. Immunol.* **139,** 1673.
8. Nacy C.A., Fortier A.H., Meltzer M.S., Buchmeier N.A. & Schreiber R.D. (1985) Macrophage activation to kill *Leishmania major*: activation of macrophages for intracellular destruction of

amastigotes can be induced by both recombinant interferon-γ and non-interferon lymphokines. *J. Immunol.* **135,** 3505.

9. SUZUKI Y., ORELLANA M.A., SCHREIBER R.D. & REMINGTON J.S. (1988) Interferon-γ: the major mediator of resistance against *Toxoplama gondii. Science*, **240,** 516.
10. NATHAN C.F., MURRAY H.W., WIEBE M.E. & RUBIN B.Y. (1983) Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. exp. Med.* **158,** 670.
11. STUEHR D.J. & MARLETTA M.A. (1987) Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. *J. Immunol.* **139,** 518.
12. NATHAN C.F. & YOSHIDA R. (1988) Cytokines: interferon-γ. In: *Inflammation: Basic Principles and Clinical Correlates* (eds J. I. Gallin, I. M. Goldstein and R. Synderman), p. 229. Raven Press, New York.
13. NIBBERING P.H., LANGERMANS J.A.M., VAN DE GEVEL J.S., VAN DER HULST M.E.B. & VAN FURTH R. (1991) Nitrite production by activated murine macrophages correlates with their toxoplasmatic activity, Ia-antigen expression, and production of H_2O_2. *Immunobiology*, **184,** 93.
14. ADAMS D.O. & HAMILTON T.A. (1987) Molecular transductional mechanisms by which IFN-γ and other signals regulate macrophage development. *Immunol. Rev.* **97,** 5.
15. KIDERLEN A.F., KAUFMANN S.H.E. & LOHMANN-MATTHES M. (1984) Protection of mice against the intracellular bacterium *Listeria monocytogenes* by recombinant immune interferon. *Eur. J. Immunol.* **14,** 964.
16. KURTZ R.S., YOUNG K.M. & CZUPRYNSKI C.J. (1989) Separate and combined effects of recombinant interleukin-1α and gamma interferon on antibacterial resistance. *Infect. Immun.* **57,** 553.
17. NAKANE A., NUMATA A., ASANO M., KOHANAWA M., CHEN Y. & MINAGAWA T. (1990) Evidence that endogenous gamma interferon is produced early in *Listeria monocytogenes* infection. *Infect. Immun.* **58,** 2386.
18. VAN FURTH R. & COHN Z.A. (1968) The origin and kinetics of mononuclear phagocytes. *J. exp. Med.* **128,** 415.
19. DIESSELHOFF-DEN DULK M.M.C. & VAN FURTH R. (1981) Characteristics of mononuclear phagocytes from different tissues. In: *Methods for Studying Mononuclear Phagocytes* (eds P. J. Edelson, H. S. Koren and D. O. Adams), p. 253. Academic Press, New York.
20. RUCH W., COOPER P.H. & BAGGIOLINI M. (1983) Assay of H_2O_2 production by macrophages and neutrophils with homovanillic acid and horse-radish peroxidase. *J. immunol. Meth.* **63,** 347.
21. VAN VLIET E., MELIS M. & VAN EWIJK W. (1984) Monoclonal antibodies to stromal cell types of the mouse thymus. *Eur. J. Immunol.* **14,** 524.
22. NIBBERING P.H., VAN DER HEIDE G.A. & VAN FURTH R. (1989) Immunocytochemical analysis of the cellular responses to BCG. *Clin exp. Immunol.* **75,** 147.
23. STEINIGER B., FALK P., LOHMÜLER M. & VAN DER MEIDE P.H. (1989) Class II MHC antigens in the rat digestive system. Normal distribution and induced expression after interferon-gamma treatment *in vivo. Immunology*, **68,** 507.
24. COHN Z.A. & MORSE S.I. (1959) Interactions between rabbit polymorphonuclear leukocytes and staphylococci. *J. exp. Med.* **110,** 419.
25. CZUPRINSKY C.J., HENSON P.M. & CAMBELL P.A. (1984) Killing of *Listeria monocytogenes* by inflammatory neutrophils and mononuclear phagocytes from immune and nonimmune mice. *J. Leukoc. Biol.* **35,** 193.
26. PORTNOY D.A., SCHREIBER R.D., CONNELLY P. & TILNEY L.G. (1989) γInterferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. *J. exp. Med.* **170,** 2141.
27. ROSEN H., GORDON S. & NORTH R.J. (1989) Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. *J. exp. Med.* **170,** 27.
28. CONLAN J.W. & NORTH R.J. (1991) Neutrophil-mediated dissolution of infected host cells as a defence strategy against a facultative intracellular bacterium. *J. exp. Med.* **174,** 741.
29. TILNEY L.G., & PORTNOY D.A. (1989) Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes. J. Cell. Biol.* **109,** 1587.
30. PORTNOY D.A., CHAKRABORTY T., GOEBEL W. & COSSART P. (1992) Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect. Immun.* **60,** 1263.
31. CURRAN R.D., BILLIAR T.R., STUEHR D.J., HOFMANN K. & SIMMONS R.L. (1989) Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. *J. exp. Med.* **170,** 1769.
32. MELLOUK S., GREEN S.J., NACY C.A. & HOFFMAN S.L. (1991) IFN-γ inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. *J. Immunol.* **146,** 3971.
33. GREGORY S.H., BARCZYNSKI L.K. & WING E.J. (1992) Effector function of hepatocytes and Kupffer cells in the resolution of systemic bacterial infections. *J. Leukoc. Biol.* **51,** 421.