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Capacity of Recombinant Gamma Interferon To Activate Macrophages for *Salmonella*-Killing Activity

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The ability of recombinant gamma interferon (rIFN-γ) to activate macrophages for *Salmonella*-killing activity was kinetically examined in relation to phagosome-lysosome fusion and H_2O_2 generation. Resident peritoneal macrophages of BALB/c mice incubated with 10^2 to 10^3 U of rIFN-γ per ml for 12 h exhibited enhanced bactericidal activity against *Salmonella typhimurium*, although H_2O_2 generation was unaltered. In contrast, macrophages incubated with equal doses of rIFN-γ for 48 h showed both an enhanced *Salmonella*-killing activity and an increased generation of H_2O_2. To evaluate *Salmonella*-killing activities of macrophages, intracellular bacteria were assayed at 0, 2, and 8 h after infection. During the initial 2 h of infection, 12-h-activated macrophages, as well as the unstimulated control macrophages, showed a decline in bacterial population at the same rate. Over the next 6 h of infection, however, the number of viable bacteria in activated macrophages remained unchanged, whereas the number of bacteria in control macrophages significantly ($P < 0.05$) increased. Similar results were obtained in 48-h-activated macrophages. On the other hand, macrophages incubated with 10 to 10^3 U of rIFN-γ exhibited enhanced fusion of lysosomes to *Salmonella*-containing phagosomes in both the 12-h- and 48-h-stimulated stages. Moreover, when 48-h-activated macrophages were incubated concomitantly with superoxide dismutase and catalase, *Salmonella*-killing activity was not affected. These results indicate that rIFN-γ per se is able to activate peritoneal macrophages to induce *Salmonella*-killing activity and suggest that increased phagosome-lysosome fusion followed by an oxygen-independent killing mechanism is primarily responsible for the enhanced *Salmonella*-killing activity in rIFN-γ-activated macrophages.

The study of typhoid immunity has been most commonly effected by using the model of *Salmonella typhimurium* infection in mice (11). The importance of cell-mediated immunity in controlling *Salmonella* infections has been well documented (7), although there has been the viewpoint that killed vaccines induce humoral immunity and can give a high level of protection against *Salmonella* infection (18, 33). One of the in vivo manifestations of cell-mediated immunity is delayed-type hypersensitivity, and its offshoot has often been correlated with protective immunity in some mouse strains (7, 8). Subsequent studies, however, have revealed that development of protective immunity has not always been associated with delayed-type hypersensitivity response (21, 36). Irrespective of controversies regarding the immune defense mechanisms for *Salmonella* infection and association of delayed-type hypersensitivity responsiveness in such mechanisms, macrophages are deemed to be the ultimate effector of host resistance to infection with *Salmonella* species. In general, the effector mechanism of cell-mediated immunity against intracellular pathogens has been established to be the enhanced bactericidal activity of macrophages activated by one of the lymphokines, macrophage-activating factor (14, 16, 26). Recently, gamma interferon (IFN-γ) has been identified as a potent macrophage-activating factor (4, 12, 32, 34, 38). Macrophages exposed to recombinant IFN-γ (rIFN-γ) develop the ability to secrete reactive oxygen intermediates (ROI), as well as to kill nonspecifically the obligate or facultative intracellular microorganisms (12, 30) and a variety of neoplastic cells (34). Similar findings have been reported for the activation of cultured human monocytes (32).

Whether the activation of macrophages by supernatants from stimulated T lymphocytes can be attributed exclusively to the action of IFN-γ is, however, still debatable (19, 22, 31). Recently, van Dissel et al. (40) concluded that, as a single activating stimulus, rIFN-γ is not capable of activating the antibacterial effector function of peritoneal macrophages against facultative intracellular pathogens such as *Listeria monocytogenes* and *S. typhimurium*.

In the present study, we provide evidence that resident peritoneal macrophages from susceptible mouse strains, when solely activated by rIFN-γ, acquired the ability to inhibit the intracellular growth of *S. typhimurium* in either short-term (12-h)- or long-term (48-h)-activated stages and that enhanced phagosome-lysosome (P-L) fusion in activated macrophages is the primary mechanism for such activity.

MATERIALS AND METHODS

Peritoneal macrophages. Male BALB/c mice (aged 8 to 12 weeks) were used throughout these studies and were obtained from Charles River Japan, Inc., Atsugi, Kanagawa.

Resident macrophages were harvested from normal mice by peritoneal lavage with Eagle minimum essential medium containing 5 U of heparin per ml. All cells were washed three times and counted with a hemacytometer. Differential cell counts showed that the peritoneal cells were mostly composed of 65% macrophages, 35% leukocytes, and <1% polymorphonuclear leukocytes.

Counting of monolayered macrophages. The number of monolayered macrophages on wells was counted by the

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method of Lissner et al. (24), using lidocaine to release macrophages from monolayers. Protein contents of the monolayers were determined by the method of Lowry et al. (25), after macrophages were lysed with 0.05% Triton X-100, and were calculated per 10^5 macrophages.

Culture medium. RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with L-glutamine and 10% fetal bovine serum (FBS; M. A. Bioproducts, Walkersville, Md.) (RPMI-FBS) was used throughout the experiments except in macrophage activation for H_2O_2 generation. A 1-ng amount of lipopolysaccharide (*Escherichia coli* O111:B4; Difco Laboratories, Detroit, Mich.) was added to RPMI-FBS.

IFN-γ. rIFN-γ (Genentech Inc., South San Francisco, Calif.) (15) was a gift from Toray Industries Inc., Tokyo, Japan. rIFN-γ was stored at 4°C in phosphate-buffered saline at a concentration of 10^6 U/ml. Its antiviral activity was assessed by a virus plaque reduction assay, using L929 cells and the vesicular stomatitis virus system.

Assay for H_2O_2. The H_2O_2-generating activity of macrophages was determined by the peroxidase-dependent oxidation of phenol red, as described by Pick and Keisari (35) with some modifications. Briefly, monolayers of resident macrophages (7.5×10^5 per well) in 24-well plates (Becton Dickinson Labware, Oxnard, Calif.) were incubated with 0.75 ml of rIFN-γ in RPMI-FBS containing 50 U of penicillin and 50 μg of streptomycin per ml. After being washed with phenol red solution (100 μg/ml) containing 17 U of horseradish peroxidase (170 U/mg of protein; Sigma Chemical Co., St. Louis, Mo.) per ml, 0.01 M potassium phosphate buffer (pH 7.0), 0.14 M NaCl, and 1 mg of glucose per ml, the monolayers were covered with 1 ml of phenol red solution containing 1 μg of phorbol myristate acetate (PMA; Sigma) per ml and incubated at 37°C in 5% CO_2–95% air for 1 h. A 10-μl portion of 1 N NaOH was added to the cell-free supernatants, and the A_{610} was determined. The results were expressed as nanomoles of H_2O_2 per milligram of macrophage protein per hour.

Bacterial strain and growth conditions. *S. typhimurium* LT-2 was used. The inoculum was prepared from an 18-h bacterial culture at 37°C in brain heart infusion broth (Difco) and quantified by determining the spectral A_{650} of a saline suspension.

Determination of macrophage *Salmonella*-killing activity. Macrophage bactericidal activity was determined by inhibition of the growth of *S. typhimurium* in macrophages after short- or long-term treatment with rIFN-γ. Briefly, monolayers of resident peritoneal macrophages (7.5×10^5 per well) in 24-well plates (Becton Dickinson Labware) were incubated with rIFN-γ in antibiotic-free RPMI-FBS in a volume of 0.75 ml at 37°C in 5% CO_2–95% air. After being washed with antibiotic-free RPMI 1640, the macrophages were infected with 2×10^6 *S. typhimurium* in the presence of 10% fresh mouse serum (total volume, 0.5 ml). The infected monolayers were washed, and some were incubated for 2 or 8 h with antibiotic-free RPMI 1640 containing 20% FBS by washing with the medium hourly to remove extracellular bacteria. Immediately following or 2 or 8 h after infection, intracellular bacteria were released by lysing the macrophages in 0.05% Triton X-100, and the population was determined by colony counting. The results were expressed as the number of bacteria per microgram of macrophage protein. The ratio of the number of viable bacteria at 8 h (Nt_8) to that at 0 h (Nt_0) was calculated, and the growth inhibition rate was determined by the following formula: percent growth inhibition = $\{1 - [(Nt_8/Nt_0 \text{ in rIFN-}\gamma\text{-treated macrophages})/(Nt_8/Nt_0 \text{ in control macrophages})]\} \times 100$.

Effects of scavengers of ROI on *Salmonella*-killing activity and reduction of NBT of macrophages. Scavengers of ROI were used at the concentrations given in reference 8. They included catalase from bovine liver recrystallized twice (2.5 mg/ml) as a scavenger of hydrogen peroxide and superoxide dismutase (SOD), type III (3,000 U/mg; 2.5 mg/ml), as a scavenger of superoxide anion. Activated macrophages were incubated for 3 h with the ROI scavengers dissolved in medium or with the medium alone before infection with *S. typhimurium* and for 8 h thereafter (6). The population of intracellular bacteria was counted at 0 and 8 h, and growth inhibition rate was determined. To determine the effect of scavengers on intracellular release of ROI, reduction of Nitro Blue Tetrazolium (NBT) was assayed. Monolayers of the peritoneal exudate cells (1.5×10^6) harvested from mice 4 h after injection with 2 ml of 0.5% glycogen on round cover slips (15 mm in diameter) were incubated with the ROI scavengers for 3 h and added with heat-killed cells of *Candida albicans* in the presence of 10% fresh mouse serum, 2 mg of NBT per ml, and the scavengers. After incubation for 90 min, the monolayers were washed with phosphate-buffered saline and dried, and formazan was extracted with 1 ml of dioxane by boiling for 30 min. The A_{580} of the extract was converted to micrograms of formazan by multiplying it by 14.14 (39).

Fluorescence assay of P-L fusion. Transfer of free acridine orange label from lysosomes to phagosomes was assayed fluoromicroscopically (3). Macrophage monolayers (7.5×10^5) on cover slips (15 mm in diameter) were preincubated with rIFN-γ as described for *Salmonella*-killing activity. After monolayers were washed with serum-free RPMI 1640 medium to remove serum components, acridine orange (5 μg/ml) was added and the cells were incubated for 15 min. The cover slips were washed free of acridine orange, infected with *S. typhimurium* for 30 min, washed to remove extracellular bacteria, and further incubated for 2 h in RPMI 1640 containing 20% FBS. After 1 h of incubation, cover slips were washed to remove extracellular bacteria. After 2 h of incubation, cover slips were washed, air dried, and tallied for the number of orange-stained phagolysosomes among 400 macrophages under a fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The same monolayers were stained with Giemsa solution to count total bacteria in macrophages. P-L fusion was expressed as the percentage of orange-stained intracellular organisms among total intracellular organisms.

RESULTS

Fate of *S. typhimurium* in peritoneal macrophages preincubated with rIFN-γ. *Salmonella*-killing activity of macrophages was assessed 12 or 48 h after preincubation of macrophages with 10^3 U of rIFN-γ. These incubation periods were selected so that the contribution of ROI to killing activity of macrophages could be determined. Representative data of three experiments are shown in Fig. 1. When the cell number and protein contents of macrophage monolayers were assayed after washing, 10^5 cells of control and activated macrophages were shown to be equivalent to 8.3 ± 0.2 and 8.9 ± 0.3 μg of protein, respectively.

The average numbers of viable *S. typhimurium* in macrophages which were preincubated with or without 10^3 U of rIFN-γ per ml for 12 h were determined at 0, 2, and 8 h after infection (Fig. 1). At 0 h, 4×10^4 to 6×10^4 viable cells were

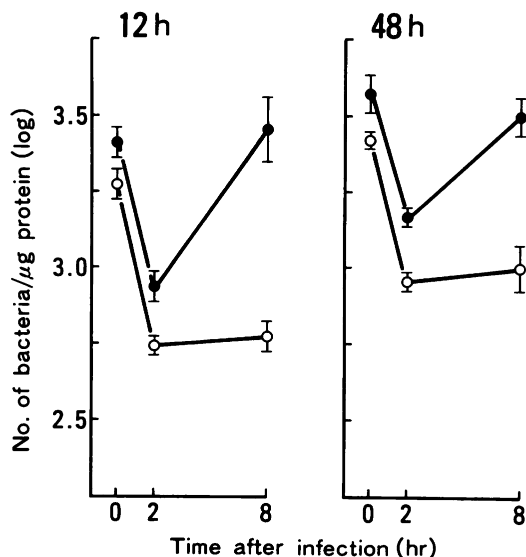

FIG. 1. Fate of *S. typhimurium* in peritoneal macrophages preincubated with rIFN-γ. Monolayers of resident peritoneal macrophages were incubated with (○) or without (●) 10^3 U of rIFN-γ per ml for 12 or 48 h and infected with *S. typhimurium* in the presence of 10% fresh serum. Immediately following or 2 or 8 h after infection, the population of intracellular bacteria was determined. Representative data of three experiments are shown.

detected in a monolayer which contained 2×10^5 to 3×10^5 macrophages as calculated from protein contents, indicating that multiplicities of infection of the macrophages were 0.23 ± 0.03 and 0.18 ± 0.04 in control and activated macrophages, respectively. The multiplicity of infection was confirmed by Giemsa staining of infected monolayers at 0 h (data not shown). During the initial 2 h of infection, macrophages showed a decline in bacterial counts compared with counts at 0 h. Although the magnitude of this initial decline in the number of viable *S. typhimurium* varied with experiment, bacterial counts at 2 h were usually <40% of those at 0 h; this percentage of reduction was similar in both control and rIFN-γ-activated macrophages. Since the loss of macrophages by this time was about 5% in both activated macrophages and the untreated control, the decrease in bacterial number is indicative of an interplay of intracellular events. Over the next 6 h of infection, viable counts of *S. typhimurium* in activated macrophages remained unchanged, whereas the number of viable bacteria in control macrophages increased 2.5-fold relative to those at 2 h. The number of bacteria present in culture fluids at 0, 2, and 8 h after infection was generally <10% of the lysate values. The ratios of the number of viable cells at 8 h to those at 0 h were 0.4 in rIFN-γ-activated macrophages and 1.2 in control macrophages. The results indicate that macrophages preincubated with 10^3 U of rIFN-γ per ml inhibit intracellular growth of *S. typhimurium* at a rate of 69%.

Macrophages were preincubated with rIFN-γ for 48 h, and the average number of viable *S. typhimurium* in macrophages was determined at 0, 2, and 8 h after infection. The multiplicities of infection of control and activated macrophages were 0.28 ± 0.03 and 0.23 ± 0.05, respectively. As evident in 12-h-activated macrophages, a marked decline in viable bacteria occurred during the initial 2 h of infection in both control and activated macrophages (Fig. 1). Over the next 6 h of infection, viable counts of *S. typhimurium* in activated macrophages remained unchanged, whereas the number of viable bacteria in control macrophages increased 2.2-fold relative to those at 2 h. The ratios of the number of viable cells at 8 h to those at 0 h were 0.4 in rIFN-γ-activated macrophages and 0.9 in control macrophages, respectively. The growth inhibition rate for *S. typhimurium* in the activated macrophages was 54%. These results indicate that rIFN-γ is able to activate macrophages for *Salmonella*-killing activity in either 12-h- or 48-h-stimulated stages.

TABLE 1. Time course of macrophage activation by rIFN-γ for H_2O_2 generation[a]

Incubation time (h)	H_2O_2 (nmol/μg of protein per h)[b]	
	Control macrophages	rIFN-γ-treated macrophages
4	23.7 ± 0.6	25.5 ± 0.3
12	25.6 ± 1.7	22.9 ± 0.9
24	25.6 ± 3.0	40.8 ± 7.4
48	22.5 ± 0.2	178.5 ± 0.5
72	24.5 ± 1.2	252.6 ± 14.6

[a] Resident macrophage monolayers were preincubated at 37°C with or without rIFN-γ (10 U/ml) for the indicated periods and their H_2O_2-generating activity was determined by triggering with 1 μg of PMA per ml. Representative data of three experiments are shown.

[b] Mean ± standard deviation of three wells.

Time course of macrophage activation by rIFN-γ for H_2O_2 generation. In assessing macrophage activation kinetics for H_2O_2 generation, resident macrophage monolayers were preincubated with rIFN-γ for 4, 12, 24, 48, or 72 h and release of H_2O_2 was measured after triggering with PMA. Macrophages preincubated with rIFN-γ for <24 h did not exhibit any increase in H_2O_2, just like the control macrophages. However, at 24 h some increase in H_2O_2 was evident, with a marked increase at 48 h; peak H_2O_2 generation occurred at 72 h. Representative data of three experiments are shown in Table 1.

Effect of rIFN-γ dose on macrophage activation for *Salmonella*-killing activity and H_2O_2 generation. The dose of rIFN-γ required to induce activation for *Salmonella*-killing activity was determined similarly to that for increased H_2O_2 generation. When macrophages were preincubated with increasing doses of rIFN-γ for 12 h, it was shown that 10^2 U/ml was required to induce a significant inhibition of intracellular growth of *S. typhimurium* during 8 h, whereas 10^3 U of rIFN-γ per ml induced greater growth inhibition in macrophages (Fig. 2). When macrophages were preincubated with rIFN-γ for 48 h, *Salmonella*-killing activity was increased dose dependently, whereupon 10^2 U/ml was required to induce significant activity (Fig. 2).

On the other hand, when macrophages were preincubated with an increased dose of rIFN-γ for 12 h, in which *Salmonella*-killing activity was enhanced dose dependently, H_2O_2-generating activity of macrophages was not enhanced in either concentration of rIFN-γ used. In contrast, 48-h-preincubated macrophages had H_2O_2-generating activities that were significantly ($P < 0.01$) enhanced by 10 U of rIFN-γ (Fig. 3). These results indicate that the rIFN-γ concentration required to induce *Salmonella*-killing activity is greater than that required for increased H_2O_2 generation and that H_2O_2 is not essential for *Salmonella*-killing activity of activated macrophages.

Effect of ROI scavengers on *Salmonella*-killing activity of long-term-activated macrophages. To determine the contribution of ROI to *Salmonella*-killing activity of long-term (48-h)-activated macrophages, in which H_2O_2-generating activity was enhanced, the effect of ROI scavengers, SOD, and

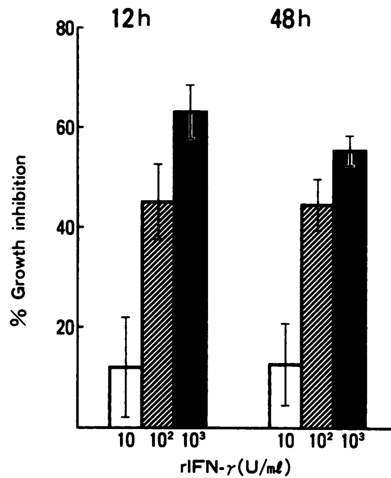

FIG. 2. Effect of rIFN-γ dose on macrophage activation for *Salmonella*-killing activity. Monolayers of resident peritoneal macrophages were incubated with three various concentrations of rIFN-γ for 12 or 48 h, and their *Salmonella*-killing activities were compared by growth inhibition. The results are expressed as the mean ± standard deviation of three experiments.

catalase on the killing activity of the macrophages was examined. Macrophages were incubated concomitantly with SOD (2.5 mg/ml) and catalase (2.5 mg/ml) for 3 h prior to infection and for 8 h thereafter. Macrophages preincubated with 10^3 U of rIFN-γ per ml for 48 h significantly inhibited the growth of intracellular bacteria in the absence of scavengers. Similarly, in scavenger-treated macrophages, the growth inhibition rate of intracellular bacteria in activated macrophages was similar to that in untreated macrophages, although growth of intracellular bacteria in both control and activated macrophages was increased. Representative data of three experiments are shown in Table 2. Moreover, to confirm the effect of scavengers on depletion of ROI in phagosomes, an NBT test was performed, using neutrophils and heat-killed cells of *C. albicans*. The results showed that NBT reduction in the presence of scavengers was decreased

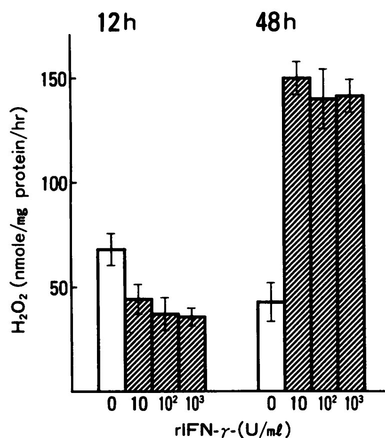

FIG. 3. Effect of rIFN-γ dose on macrophage activation for H_2O_2-generating activity. Monolayers of resident peritoneal macrophages were incubated with rIFN-γ for 12 or 48 h, and their H_2O_2-generating activities were determined by triggering with 1 μg of PMA per ml. Representative data of three experiments are shown.

TABLE 2. Effect of ROI scavengers on *Salmonella*-killing activity of long-term-activated macrophages

Scavengers[a]	Macrophages preincubated with[b]:	No. of bacteria/μg of protein: 0 h	No. of bacteria/μg of protein: 8 h	Nt_8/Nt_0[c]	% Growth inhibition
−	Medium	6,514 ± 84	11,809 ± 540	1.8	
	rIFN-γ	3,491 ± 487	1,047 ± 98	0.3	83
+	Medium	5,283 ± 542	52,820 ± 7,022	10.0	
	rIFN-γ	3,366 ± 365	5,386 ± 679	1.6	84

[a] Catalase (2.5 mg/ml) and SOD (2.5 mg/ml) were used concomitantly.
[b] Macrophages were preincubated with or without 10^3 U of rIFN-γ per ml for 48 h.
[c] Nt_0 and Nt_8 indicate bacterial numbers at 0 and at 8 h, respectively.

by >50% compared with reduction of NBT in the absence of the scavengers, implying that scavengers were able to act in phagosomes to deplete ROI to a significant extent (Table 3). These results indicate that increased *Salmonella*-killing activity in long-term-activated macrophages is not caused by the enhanced generation of ROI.

Effect of rIFN-γ on P-L fusion after phagocytosis of *S. typhimurium*. Macrophage monolayers preincubated with 10 to 10^3 U of rIFN-γ per ml for 12 or 48 h were treated with acridine orange and infected with *S. typhimurium*, and P-L fusion in the macrophages was determined. Phagosomes fused with lysosomes were stained orange by transfer of acridine orange label in lysosomes (Fig. 4). In macrophages preincubated with rIFN-γ for 12 h, the P-L fusion was significantly increased; the stimulation index was >5.7. Macrophages preincubated with rIFN-γ for 48 h also exhibited enhanced P-L fusion, whereas the rate of stimulation was lower than that in 12-h-stimulated macrophages. Increased rIFN-γ concentration did not alter the rate of P-L fusion (Table 4). These results indicate that the time course of P-L fusion coincides well with that of the activation of macrophages for *Salmonella*-killing activity and suggest that enhanced *Salmonella*-killing activity in rIFN-γ-activated macrophages is attributable to enhanced P-L fusion.

DISCUSSION

In the present experiments, the kinetics and capacities of macrophage activation by rIFN-γ for *Salmonella*-killing activity vis-à-vis P-L fusion and H_2O_2 generation are documented.

Whether activation of macrophages for *Salmonella*-killing activity requires the series of stages observed in macrophage-mediated tumor cytotoxicity (17) or not remains unsettled. Nonetheless, we added 1 ng of lipopolysaccharide per ml to the medium in each experimental system.

TABLE 3. Effect of ROI scavengers on NBT reduction in phagosomes of neutrophils after phagocytizing heat-killed cells of *C. albicans*

Ratio of *C. albicans*/neutrophils	Formazan (μg/90 min per 1.5×10^6 cells): Without scavengers	Formazan (μg/90 min per 1.5×10^6 cells): With scavengers	% Reduction
4:1	3.01 ± 0.61	1.52 ± 0.14[a]	50
0.4:1	0.75 ± 0.17	0.28 ± 0.15[b]	68

[a] $P < 0.01$.
[b] $P < 0.05$.

FIG. 4. Fluorescent photomicrograph of acridine orange-labeled activated macrophages infected with *S. typhimurium*. Monolayers of resident peritoneal macrophages were incubated with 10^3 U of rIFN-γ per ml for 12 h, labeled with acridine orange, and infected with *S. typhimurium*. The monolayers were dried 2 h after infection. An acridine orange-stained phagosome is observed in the macrophage on the left.

Lissner et al. (24) examined *Salmonella*-killing activity of resident macrophages from a genetically distinct strain of mice for 24 h, using gentamicin as an extracellular growth inhibitor. However, since pinocytic activity of activated macrophages has been demonstrably enhanced (10), the possibility that more antibiotics are infused into activated macrophages, thereby eliciting an artifact, cannot be ignored. Therefore, we performed macrophage activation and intracellular bactericidal assays without any addition of antibiotics. In the latter case, routine washings were performed hourly to remove extracellular bacteria. A low multiplicity of infection in our phagocytic system suggests that bacteria can be completely phagocytized in both activated macrophages and the untreated control. Therefore, the possibility that some of the increase represents extracellular growth of unphagocytized bacteria on the surface of macrophages could be ruled out.

Although several reports have emerged regarding the ability of rIFN-γ to enhance the antimicrobial activity of macrophages against various pathogens such as *Mycobacterium tuberculosis* (12), *Toxoplasma gondii* (32), *Leishmania donovani* and *Trypanosoma cruzi* (30), and *C. albicans* and *Blastomyces dermatitidis* (5), little has yet been reported on the ability of rIFN-γ to activate macrophages for *Salmonella*-killing activity. Recently, van Dissel et al. (40) contended that rIFN-γ failed to induce a state of activation in macrophages for killing of ingested *Listeria* and *Salmonella* spp., in vitro or in vivo. Our findings, however, showed that 10^2 to 10^3 U of rIFN-γ per ml activated macrophages for bactericidal activity against *S. typhimurium* when the assay was counting of viable cells 8 h after infection. The apparent difference in evaluation of activities in vitro is that we observed killing activity for 8 h after injection, whereas van Dissel et al. observed it for only 1 h after infection. Two hours after infection, ingested live bacteria in both control and activated macrophages declined at the same rate as reported by van Dissel et al. (40), although the precise mechanism is still undetermined. It was clearly shown in this study that intracellular bacteria markedly proliferated in control macrophages, but they were statistically unchanged in activated macrophages after the next 6 h of infection.

TABLE 4. Effect of rIFN-γ on macrophage activation for enhanced P-L fusion after phagocytosis of *S. typhimurium*

Preincubation time (h)	rIFN-γ (U/ml)	Fusion (%)	Stimulation index
12	0	4.6 ± 2.2	
	10	26.7 ± 5.0[a]	5.8
	100	26.1 ± 3.3[a]	5.7
	1,000	31.7 ± 6.9[a]	6.9
48	0	4.0 ± 2.5	
	10	12.3 ± 3.9[b]	3.1
	100	14.0 ± 4.0[a]	3.5
	1,000	18.4 ± 3.2[a]	4.6

[a] $P < 0.01$.
[b] $P < 0.05$.

It has been well established that one of the antimicrobial mechanisms of activated macrophages is the production and intracellular release of ROI. The ability of activated macrophages to kill species of *Leishmania* (30) and *Toxoplasma* (32) is very closely coupled with their capacity for PMA-triggered release of ROI. However, our present findings show that *Salmonella*-killing activity was enhanced when macrophages were activated with rIFN-γ for 12 h, during which time increased generation of H_2O_2 above the normal limit was not detected. Several agents have been used to deplete the ROI to evaluate the oxygen-dependent killing mechanism in macrophages against protozoa (6, 28). When 48-h-activated macrophages were concomitantly treated with SOD and catalase, *Salmonella*-killing activity was totally unaffected, even though the effective dose of the scavengers that reputedly suppressed intracellular killing of protozoa in activated macrophages had been used (6). Furthermore, the effect of scavengers on depletion of intracellular release of ROI in the assay for *Salmonella* killing has been verified by measuring formazan, which has been formed by reducing NBT with ROI, in extracts of neutrophils after phagocytizing yeast cells together with scavengers and NBT. Since the NBT test was not sensitive enough to detect miniscule doses of ROI released in activated macrophages after phagocytizing *S. typhimurium*, we applied a system of phagocytosis between neutrophils and *C. albicans*. Although depletion of ROI in phagosomes was not wholly effected by scavengers, the depletion rate (roughly 50%) is ostensibly sufficient to affect oxygen-dependent killing in phagocytes, as reduction of NBT in phagosomes is not always caused by ROI (2). These results suggest that an oxygen-independent mechanism primarily contributes to the killing of *Salmonella* spp. in activated macrophages as reported in intracellular killing of protozoa in lymphokine-activated murine (26) and rat alveolar (6) macrophages, *Chlamydia psittaci* in IFN-γ-activated murine and human macrophages (29, 37), and *Mycobacterium bovis* in rIFN-γ-activated bone marrow-derived murine macrophages (13).

Some obligate or facultative intracellular microbes are known to inhibit P-L fusion (1, 20). Enhanced P-L fusion was shown in lymphokine-activated macrophages against *Coccidioides immitis* in association with enhanced killing activity (3). On the other hand, it was reported that *S. typhimurium* displays intracellular parasitism not by preventing P-L fusion but by resisting lysosomal enzymes (9). In this study, however, phagosomes containing *S. typhimurium* did not readily fuse with lysosomes in resident macrophages, and a significant increase in P-L fusion was shown in

macrophages activated by rIFN-γ, leading to an enhanced *Salmonella*-killing activity in the macrophages. These results suggest that increase of P-L fusion is closely coupled to enhanced *Salmonella*-killing activity in activated macrophages. Flesch and Kaufmann have suggested that P-L fusion is involved in the growth inhibition of *M. bovis* by rIFN-γ-activated macrophages by using agents that modulate P-L fusion (13). It has been reported that results of P-L fusion with acridine orange as a lysosome marker must be interpreted with caution; acridine orange is evidently recognizable only in phagolysosomes in which microorganisms have been killed or severely injured (27). Our results obtained 2 h after infection showed that although the rate of orange-stained phagolysosomes was increased in activated macrophages, ingested live bacteria in both control and activated macrophages declined at the same rate. Thus, enhanced P-L fusion observed in activated macrophages is thought to induce enhanced killing in activated macrophages.

To activate macrophages for *Salmonella*-killing activity, 10^2 to 10^3 U of rIFN-γ per ml was required, while only 10 U of rIFN-γ per ml was required for P-L fusion, although increased P-L fusion was correlated kinetically to *Salmonella*-killing activity of activated macrophages. We thus postulate that generation of increased killing factor(s) in lysosomes may require a higher dose of rIFN-γ than induction of P-L fusion.

A strain of *S. typhimurium* was shown to be killed in either short-term (12-h)- or long-term (48-h)-activated macrophages. However, our preliminary experiments reveal that this does not hold true for other facultative intracellular microbes, i.e., *C. albicans* and *L. monocytogenes*, which were killed only in short-term-activated macrophages (unpublished data). We postulate that these complexities of microbicidal activity in activated macrophages may be due to different effector molecules which vary in induction and the regulation mechanisms necessary for killing of each intracellular pathogen (23). Further studies will focus on elucidating the structure and action of these molecules.

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