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Effect of macrophage activation on killing of *Listeria monocytogenes*. Roles of reactive oxygen or nitrogen intermediates, rate of phagocytosis, and retention of bacteria in endosomes

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SUMMARY

The role of macrophage activation in the killing of *L. monocytogenes* is unclear. Some studies suggest that activation for enhanced production of reactive oxygen and nitrogen intermediates may not be of central importance. Recent data have indicated an important role for interferon-gamma (IFN-γ) induced retention of *L. monocytogenes* in endosomes. Data from the present study indicate that proteose peptone-elicited macrophages from DBA2/J, CD-1, and C3H/HeN mice are listericidal. Activation of these cells *in vitro* for 20 h by IFN-γ (20 or 500 U/ml) increased H_2O_2 or nitrite production, but did not increase the number of *L. monocytogenes* killed during a subsequent 6-h or 7-h culture. Incubation of macrophages with IFN-γ plus lipopolysaccharide (LPS) caused greater activation and increased the number of Listeria killed during a 6-h or 7-h culture. However, this seems primarily attributable to enhanced phagocytosis. Proteose peptone-elicited macrophages were significantly more effective than resident macrophages in preventing the escape of *L. monocytogenes* from endosomes into the cytoplasm. This capability was not significantly enhanced by IFN-γ *in vitro*, but was enhanced by IFN-γ plus LPS. This correlates well with the effects of these activation stimuli on killing of *L. monocytogenes* by proteose peptone-elicited macrophages. These results indicate that enhanced retention of *L. monocytogens* in endosomes is induced by proteose peptone elicitation and that further macrophage activation *in vitro* by IFN-γ does not improve listericidal activity.

Keywords macrophage activation *Listeria monocytogenes* interferon-gamma retention in endosomes

INTRODUCTION

Macrophages occupy a central position in the immune system. Not only are they critical in acquired immune responses because of their role as antigen-processing and antigen-presenting cells, but they are also important in innate immunity by virtue of their phagocytic and anti-microbial capabilities. Macrophages exhibit several levels of activation that impart differing functional capacities. Resident macrophages from uninfected animals are generally considered unactivated. These cells can be primed by exposure to interferon-gamma (IFN-γ), and primed cells can be fully activated for tumouricidal function by subsequent exposure to bacterial lipopolysaccharide (LPS) [1,2]. Macrophage anti-microbial activity varies with different types of microbes, but it is generally thought to be increased as the macrophage moves further along the activation continuum [1,2].

However, the role of macrophage activation in the killing of facultative intracellular pathogens such as *L. monocytogenes* and *Salmonella typhimurium* is not clear. Several recent studies suggest that activation may not improve the ability of macrophages to kill such bacteria [3,4]. In one study, the ability of resident and proteose peptone-elicited macrophages to kill *L. monocytogenes* and *S. typhimurium* did not increase following activation *in vitro* with IFN-γ [4]. In another study, macrophage activation with IFN-γ, with or without LPS, did not enhance listericidal activity, and acquisition of tumouricidal activity was actually associated with loss of listericidal activity [3]. However, when Kagaya *et al.* [5] measured anti-bacterial activity *in vitro* for 8 h instead of 1–3 h [3,4], enhanced bactericidal activity was observed. Enhanced *in vitro* killing of *L. monocytogenes* has also been reported after activation of macrophages *in vivo* [6]. Thus, uncertainty remains as to the relationship between macrophage activation status and ability to kill intracellular pathogenic bacteria. A clear understanding of this relationship is particularly important in view of recent reports suggesting that a key component in host resistance to *L. monocytogenes* is the IFN-γ-induced ability to retain these bacteria in endosomes and prevent their escape to the cytoplasm where they are able to

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grow [7]. It is unclear whether concomitant activation of classical anti-microbial mechanisms such as the generation of reactive oxygen or nitrogen intermediates is also required for the anti-listerial activity associated with increased retention of bacteria in endosomes [7]. In addition, little is known regarding the level of macrophage activation required to induce enhanced retention in endosomes. The present study was conducted to investigate these issues.

MATERIALS AND METHODS

Mice

Mice were maintained on 12 h day/night cycles in a humidity- and temperature-controlled environment. They were given food (Purina Lab Chow) and water *ad libitum*. Female mice aged 8–14 weeks were used. C3H/HeN and CD-1 mice were purchased from Charles River Breeding Laboratories and DBA2/J mice were purchased from Jackson Laboratories.

Reagents

Recombinant murine IFN-γ (0·001 ng of endotoxin per $2{\cdot}0 \times 10^6$ U of interferon) was generously provided by Genentech (San Francisco, CA). RPMI 1640 medium was purchased from GIBCO Laboratories (Grand Island, NY). Fetal calf serum (FCS) was purchased from HyClone Laboratories (Logan, UT). LPS (phenol extract of *Escherichia coli* strain B 0111), HBSS, horseradish peroxidase (HRPO, type II, salt-free powder), phenolphthalein (phenol red, sodium salt), phorbol 12-myristate 13-acetate (PMA), hydrogen peroxide (30% solution), and endotoxin-free water were purchased from Sigma Chemical Co. (St Louis, MO). Proteose peptone was purchased from Difco Laboratories (Detroit, MI).

Isolation and culture of macrophages

Proteose peptone-elicited macrophages were induced 3 days before harvesting by injecting 1 ml of a sterile 10% proteose peptone solution intraperitoneally. All mice were then killed with CO_2. The peritoneal cavity was lavaged with ice-cold complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin, and 100 μg/ml streptomycin, pH 6·8). Cells were pooled from groups of 15–20 mice, centrifuged at 300 ***g***, and resuspended to the appropriate cell density, and plated in 96-well microtitre plates. Macrophages/ml plated were determined by differential leucocyte counts. After four washes to remove non-adherent cells, adherent populations were determined by differential staining to be greater than 95% macrophages. All macrophage cultures were incubated in a humidified incubator at 37°C with 5% CO_2–95% air.

Bacteria

Listeria monocytogenes (strain EGD from the American Type Culture Collection) was used in all bactericidal assays. This strain was selected on the basis of its use by other investigators in studies similar to those reported here [3,4]. Bacteria were grown to mid-log phase in Todd Hewitt broth, divided into small portions, and stored at −80°C using 10% glycerol as a cryoprotective agent. A growth curve was constructed using the optical density at 550 nm compared to colony-forming units (CFU) as determined by the spread plate method. For each experiment, organisms were grown to mid-log phase in Todd Hewitt broth and recovered by centrifugation. Cells were then washed twice with HBSS and resuspended in RPMI 1640 culture medium without antibiotics at the desired cell density.

In vitro *activation of macrophages*

Triplicate wells were used in all cultures. Control macrophages received only culture medium. IFN-γ was used at 500 U/ml or 20 U/ml with or without 10 ng/ml LPS. Macrophages were cultured in complete medium with interferon or interferon plus LPS for approximately 20 h. All macrophage activational parameters were assessed with the same population of macrophages used in the listericidal assay.

Bactericidal assay

To measure listericidal activity, macrophages were plated in 96-well microtitre plates at $1{\cdot}5 \times 10^6$ cells/ml (200 μl/well) for 2 h, washed, then incubated for 20 h with the activation stimuli indicated above. Macrophages were washed twice with HBSS and then washed twice with culture medium containing no antibiotics. Live *L. monocytogenes* ($1{\cdot}0 \times 10^7$ CFU/ml in 200 μl RPMI 1640 with 20% normal mouse serum) were added to cultured macrophages. Culture plates were centrifuged at 100 ***g*** for 5 min to accelerate bacterial sedimentation. Two hours after the addition of bacteria, macrophages were washed four times with antibiotic-free RPMI 1640 to remove extracellular bacteria and one set of macrophages was lysed by the addition of 200 μl distilled water to each well. *L. monocytogenes* that were released were counted using the spread plate method. This procedure was repeated with a second set of macrophages 6 or 7 h after the first set. This method was adapted from Kagaya *et al.* [5].

Phagocytosis assay

Proteose peptone-elicited and resident macrophages from CD-1 mice were isolated as described above. Macrophages were plated at $1{\cdot}5 \times 10^6$ macrophages/ml in 200 μl of complete medium. Macrophages were treated with complete medium in control cultures, 20 U/ml IFN-γ, or 20 U/ml IFN-γ plus 10 ng/ml LPS for 20 h. The macrophages were then washed extensively to remove antibiotics and activating agents and $1{\cdot}0 \times 10^7$ CFU/ml of *L. monocytogenes* were added to each well in 200 μl of antibiotic-free complete medium. Four hours later macrophages were removed from 96-well culture plates with a non-enzymatic cell dissociation solution (Sigma Chemical Co.), cytocentrifuged, and stained with Wright's stain. One-hundred macrophages per slide were counted and the number of *L. monocytogenes* per macrophage were counted.

Assays to assess activation status

Hydrogen peroxide production was measured using the phenol red method of Pick & Mizel [8]. Briefly, $4{\cdot}0 \times 10^6$ macrophages/ml (200 μl/well) were plated in microtitre plates and activated as indicated above for 20 h. Macrophages were washed three times with HBSS and resuspended in 200 μl of HBSS containing 0·56 mM phenol red and 20 U/ml HRPO. The respiratory burst was triggered with 200 nM PMA for 1 h. The oxidation of phenol red was stopped with the addition of 10 μl of 1 *N* NaOH and the absorbance of culture supernates at 610 nm was measured with a plate reader. Absorbance values were compared with a standard curve established at the same time using known concentrations of hydrogen peroxide.

Table 1. Comparison of numbers of *L. monocytogenes* killed by macrophages in culture

Treatment	Experiment no.	Strain	*L. monocytogenes* (CFU/ml) Initial*	Final*	Final-Initial	95% confidence interval (CFU/ml final–initial)
Control	1	C3H/HeN	$8{\cdot}8\times10^5\pm3{\cdot}1\times10^4$	$9{\cdot}7\times10^4\pm2{\cdot}7\times10^4$	$-7{\cdot}8\times10^5$	$-8{\cdot}6\times10^5$ to $-7{\cdot}1\times10^5$
IFN 500†			$4{\cdot}2\times10^5\pm3{\cdot}0\times10^4$	$2{\cdot}6\times10^4\pm8{\cdot}5\times10^3$	$-3{\cdot}9\times10^5$	$-4{\cdot}7\times10^5$ to $-3{\cdot}2\times10^5$
IFN 500+LPS‡			$1{\cdot}7\times10^6\pm2{\cdot}0\times10^4$§	$5{\cdot}3\times10^5\pm1{\cdot}1\times10^5$	$-1{\cdot}2\times10^6$	$-1{\cdot}7\times10^6$ to $-6{\cdot}7\times10^5$
Control	2	DBA/2J	$1{\cdot}4\times10^6\pm1{\cdot}9\times10^5$	$1{\cdot}7\times10^5\pm2{\cdot}7\times10^4$	$-1{\cdot}2\times10^6$	$-1{\cdot}5\times10^6$ to $-9{\cdot}2\times10^5$
IFN 500			$1{\cdot}5\times10^6\pm4{\cdot}0\times10^5$	$3{\cdot}8\times10^5\pm1{\cdot}3\times10^5$	$-1{\cdot}1\times10^6$	$-1{\cdot}8\times10^6$ to $-4{\cdot}0\times10^6$
IFN 500+LPS			$4{\cdot}6\times10^6\pm3{\cdot}5\times10^5$§	$2{\cdot}0\times10^6\pm6{\cdot}1\times10^5$§	$-2{\cdot}6\times10^6$	$-3{\cdot}7\times10^6$ to $-1{\cdot}4\times10^6$
Control	3	CD-1	$3{\cdot}4\times10^6\pm3{\cdot}2\times10^5$	$5{\cdot}5\times10^5\pm1{\cdot}3\times10^5$	$-2{\cdot}8\times10^6$	$-3{\cdot}4\times10^6$ to $-2{\cdot}3\times10^6$
IFN 500			$3{\cdot}2\times10^6\pm3{\cdot}6\times10^5$	$7{\cdot}5\times10^5\pm1{\cdot}3\times10^5$	$-2{\cdot}4\times10^6$	$-3{\cdot}0\times10^6$ to $-1{\cdot}8\times10^6$
IFN 20			$3{\cdot}1\times10^6\pm2{\cdot}8\times10^5$	$6{\cdot}3\times10^5\pm7{\cdot}0\times10^4$	$-2{\cdot}5\times10^6$	$-2{\cdot}9\times10^6$ to $-2{\cdot}0\times10^6$
IFN 500+LPS			$7{\cdot}0\times10^6\pm9{\cdot}4\times10^5$§	$2{\cdot}3\times10^6\pm4{\cdot}5\times10^5$§	$-4{\cdot}6\times10^6$	$-6{\cdot}3\times10^6$ to $-3{\cdot}0\times10^6$
IFN 20+LPS			$8{\cdot}4\times10^6\pm5{\cdot}8\times10^5$§	$4{\cdot}6\times10^6\pm5{\cdot}3\times10^5$§	$-6{\cdot}0\times10^6$	$-7{\cdot}3\times10^6$ to $-4{\cdot}8\times10^6$
Control	4	CD-1	$3{\cdot}1\times10^6\pm1{\cdot}2\times10^5$	$2{\cdot}8\times10^6\pm2{\cdot}8\times10^5$	$-3{\cdot}2\times10^5$	$-8{\cdot}1\times10^5$ to $-1{\cdot}7\times10^5$
IFN 500			$3{\cdot}1\times10^6\pm2{\cdot}4\times10^5$	$1{\cdot}9\times10^6\pm2{\cdot}1\times10^5$§	$-1{\cdot}2\times10^6$	$-1{\cdot}7\times10^6$ to $-7{\cdot}0\times10^5$
IFN 20			$2{\cdot}2\times10^6\pm2{\cdot}2\times10^5$§	$2{\cdot}0\times10^6\pm3{\cdot}6\times10^5$§	$-2{\cdot}4\times10^5$	$-9{\cdot}1\times10^5$ to $-4{\cdot}4\times10^5$
IFN 500+LPS			$4{\cdot}1\times10^6\pm2{\cdot}0\times10^5$§	$1{\cdot}5\times10^6\pm3{\cdot}5\times10^4$§	$-2{\cdot}6\times10^6$	$-2{\cdot}9\times10^6$ to $-2{\cdot}2\times10^6$
IFN 20+LPS			$3{\cdot}9\times10^6\pm5{\cdot}9\times10^5$§	$1{\cdot}8\times10^6\pm1{\cdot}4\times10^5$§	$-2{\cdot}1\times10^6$	$-3{\cdot}0\times10^6$ to $-1{\cdot}1\times10^6$

* Values are means ± s.e.m. for triplicate cultures, except for the final time-point of experiment 1 in which duplicate samples were used. Final–Initial values indicate the number of listeria killed during the culture period.

† IFN 500, 500 U/ml IFN-γ; IFN 20, 20 U/ml IFN-γ. Macrophages were incubated with activating stimuli for 20 h and washed before addition of *L. monocytogenes*.

‡ IFN + LPS, IFN-γ plus 10 ng/ml LPS.

§ Values significantly different from control ($P<0{\cdot}05$ by Dunnett's test). Because $n=2$ was insufficient for statistical calculations, statistical analysis was not performed for the final time-point in experiment 1.

Nitrite production was measured using the Greiss reagent as reported by Ding *et al.* [9]. Briefly, $1{\cdot}5\times10^6$ macrophages/ml (in 200 μl complete medium) were plated in microtitre plates and treated as indicated above for 20 h. One-hundred microlitres of culture supernatant were removed and added to 100 μl of Greiss reagent. Absorbance at 550 nm was determined and compared with a standard curve prepared at the same time using known concentrations of sodium nitrite.

Tumour cytotoxicity was assessed by measuring ^{51}Cr release from P815 tumour cells as described by Friedman & Beller [10]. Briefly, macrophages at $4{\cdot}0\times10^6$, $2{\cdot}0\times10^6$, and $1{\cdot}0\times10^6$ cell/ml in 100 μl of medium were treated as indicated above for 4 h. Labelled P815 cells at $6{\cdot}0\times10^5$ cells/ml in 100 μl were added to each well for 16 h, then 100 μl of supernatant were removed and counted with a gamma counter.

Transmission electron microscopy

Resident and proteose peptone-elicited peritoneal macrophages were cultured for 20 h, then infected with *L. monocytogenes* for 4 h. Macrophages were removed from 96-well microtitre plates with a non-enzymatic cell dissociation solution (Sigma Chemical Co.). Cells were centrifuged at 200 *g* for 15 min. Cell pellets were fixed in McDowell's phosphate buffered 4% formaldehyde–1% glutaraldehyde for at least 2 h. Fixed cells were washed in 0·1 M phosphate buffer (pH 7·2) and post-fixed in 1% osmium tetroxide in 0·1 M phosphate buffer for 1 h. After washing with 0·1 M phosphate buffer, cells were dehydrated in a series of graded acetones and embedded with an epoxy resin mixture. Blocks were sectioned into 70-nm ultra-thin sections with a diamond knife. Sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (Jeol, JEM-100CXII).

Statistical analysis

Assays were performed using triplicate cultures, unless otherwise noted. Analysis of variance (ANOVA) was used to determine whether any differences between groups were significant at the 0·05 level. Duncan's new multiple range test or Dunnett's test was used to separate the means. Student's *t*-test was used to determine the 95% confidence intervals for differences between numbers of bacteria killed at the initial time-point and the final time-point. The percentage of bacteria in the cytoplasm *versus* the percentage of bacteria in the endosome was evaluated using Zar's method of multiple comparisons of proportions [11].

RESULTS

Listericidal activity and phagocytic activity

The Listericidal assay used in this study involves an initial 2-h incubation of bacteria with macrophages during which phagocytosis, and presumably some killing of bacteria, occurs. This is followed by extensive washing to remove extracellular bacteria and a 6-h or 7-h incubation during which intracellular killing (or growth) of bacteria can occur.

Data shown in Table 1 indicate the initial (2 h after addition of bacteria) and final (8 h after addition of bacteria in experiment 2 and 9 h after in the other experiments) numbers of viable *L. monocytogenes* in macrophages activated *in vitro* with

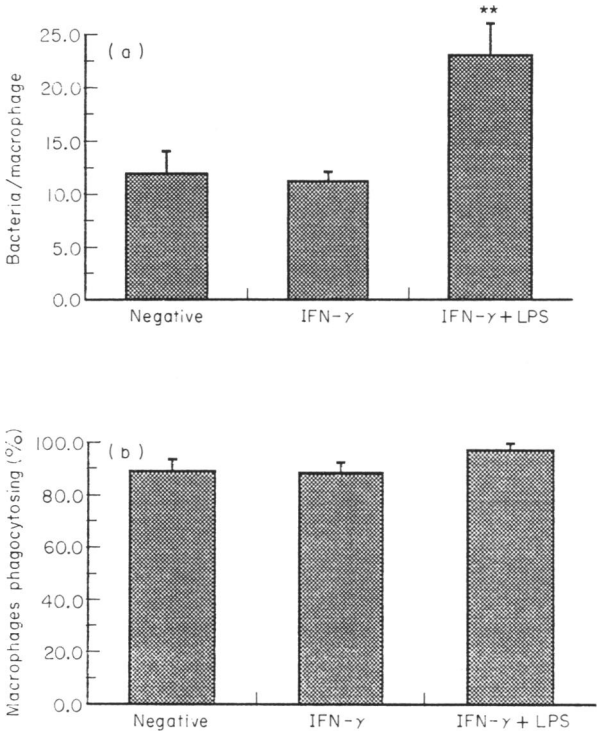


Fig. 1. Phagocytic activity of proteose peptone-elicited macrophages from CD-1. Macrophages were activated *in vitro* for 20 h with 20 U/ml IFN-γ with or without 10 ng/ml LPS. The macrophages were then washed with antibiotic-free complete medium and live *L. monocytogenes* were added to each well. Culture plates were centrifuged at 100 ***g*** for 5 min then incubated for 1 h at 37°C. Macrophages were removed from culture plates with a non-enzymatic disassociation solution then cytocentrifuged and stained with Wright's stain. One-hundred macrophages per slide were counted, and the number of *L. monocytogenes* per macrophage were counted. Values shown are means ± s.e.m. of triplicate cultures. **Indicates significant difference from control ($P < 0{\cdot}01$ by ANOVA and Dunnett's test).

IFN-γ or IFN-γ plus LPS. The number of *L. monocytogenes* killed between the initial and final time-points was calculated by subtracting the mean number of bacteria at the initial time-point from the number present at the final time-point. These data are shown in Table 1, and the negative values obtained in all cultures indicate that *L. monocytogenes* were killed by control and activated macrophages from all three mouse strains used in this study. Comparing the mean values of control cultures to the 95% confidence intervals for cultures activated with IFN-γ indicates that IFN-γ did not significantly increase the number of *L. monocytogenes* killed. Experiment 4 was the only exception. In this experiment, IFN-γ significantly increased the number of bacteria killed, but this was only observed at an IFN-γ concentration of 500 U/ml (Table 1). IFN-γ plus LPS significantly increased the number of *L. monocytogenes* killed during the culture period. However, IFN-γ plus LPS also increased the number of bacteria within macrophages at both the initial and final time-points compared with the number in untreated (control) macrophages. In contrast, IFN-γ alone did not increase the number of bacteria at the initial or final time points (Table 1).

It has been reported that LPS enhances phagocytosis [12], and therefore it seemed likely that the increased number of

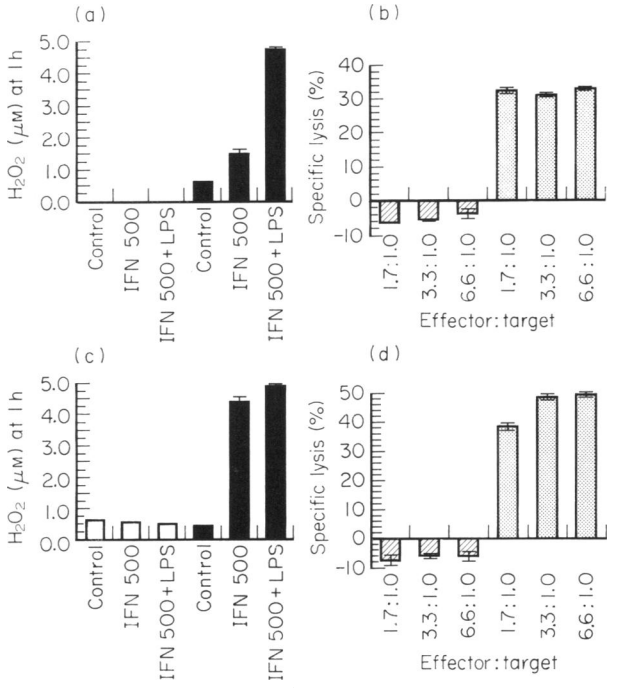


Fig. 2. Production of H_2O_2 and tumour cytotoxicity as indicators of macrophage activation. Macrophages from the same populations of cells used in experiments 1 and 2 in Table 1 were used in these assays. Macrophages were activated for 20 h (IFN 20, IFN-γ at 20 U/ml; IFN 500, IFN-γ at 500 U/ml; LPS, LPS at 10 ng/ml). Control cultures received culture medium only. After washing to remove the activating agents, the respiratory burst was triggered with 200 nM PMA in some cultures and DMSO (vehicle for PMA) was added to others (Negative). Hydrogen peroxide production was measured by the horseradish-peroxidase-catalysed reduction of phenol red as detected spectrophotometrically. For assessment of tumor cytotoxicity, macrophages were treated with culture medium (Control) or IFN-γ at 500 U/ml plus LPS at 10 ng/ml for 4 h and ^{51}Cr-labelled P815 tumour cells were added to each group of macrophages. Sixteen hours later, 100 μl of culture supernatant were removed and counted on a gamma counter. All treatments were performed in triplicate, and values shown are means ± s.e.m. (a) (b) Experiment 1, C3H/HeN mice; (c) (d) experiment 2, DBA/2J mice; □, negative, ■, PMA; ■, control; ▨ IFN 500 + LPS.

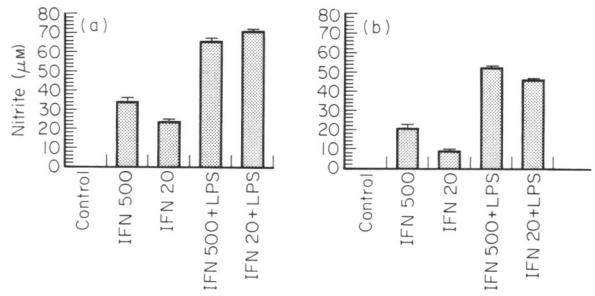


Fig. 3. Nitrite production as an indicator of macrophage activation. Proteose peptone-elicited macrophages from the same populations of cells used in experiments 3 (a) and 4 (b) in Table 1 were used to assess nitrite production. Macrophages were activated as described in Fig. 2. After 20 h, 100 μl of culture supernatant were removed and added to 100 μl of Greiss reagent and absorbance was measured using a multi-channel plate reader and compared to a standard curve. All cultures were performed in triplicate, and values shown are means ± s.e.m.

Table 2. Retention of *L. monocytogenes* in endosomes in resident and proteose peptone-elicited macrophages after *in vitro* activation with IFN-γ or IFN-γ plus LPS

Treatment *in vitro**	Listeria counted†	Listeria in endosomes (%)	P‡
Resident			
Control	32	46·7	
IFN-γ	28	75·7	<0·001
IFN-γ+LPS	40	88·9	<0·001
Proteose peptone			
Control	37	67·6	
IFN-γ	44	79·6	>0·05
IFN-γ+LPS	49	93·9	<0·001

* Peritoneal macrophages from CD-1 mice were plated at $1{\cdot}5 \times 10^6$/ml, washed to remove non-adherent cells, and incubated for 20 h with IFN-γ (20 U/ml) or IFN-γ (20 U/ml) plus LPS (10 ng/ml). Live Listeria were added after washing, and macrophages were harvested for electron microscopy 4 h later.

† Values shown are the total number of Listeria counted in each group.

‡ Control values were compared to treatment values using a method for multiple comparisons of proportions [11]. Resident control and proteose peptone control values were also compared and were significantly different ($P<0{\cdot}01$).

bacteria found in macrophages activated with IFN-γ plus LPS was caused by enhanced phagocytosis. The data shown in Fig. 1 indicate that macrophages activated with IFN-γ plus LPS phagocytose roughly two-fold more *L. monocytogenes* than macrophages activated with IFN-γ alone or control macrophages. These data also indicate that the increase in phagocytosis was caused by increased uptake of *L. monocytogenes* and not an increase in percentage of macrophages phagocytosing.

Activation status

Macrophages from the same populations used for the listericidal assays shown in Table 1 were evaluated for H_2O_2 production, tumour cytotoxicity, or nitrite production (Figs 2 and 3). These assays demonstrate that the macrophages were activated by IFN-γ alone and that a higher level of activation was generally achieved using IFN-γ plus LPS. Macrophages activated with 500 U/ml IFN-γ plus 10 ng/ml LPS were cytotoxic for tumour cells, indicating a high level of activation.

Retention of Listeria in endosomes

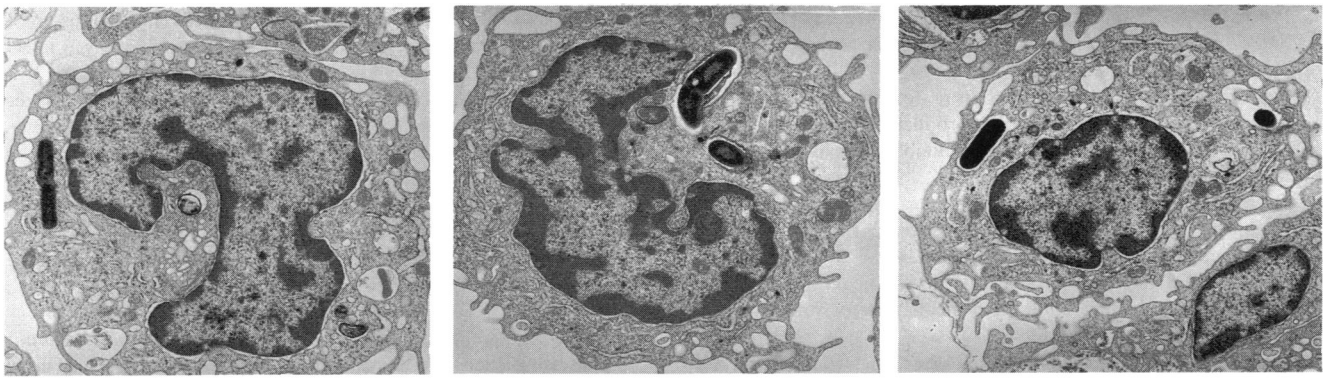
Resident macrophages treated for 20 h *in vitro* with IFN-γ alone or IFN-γ plus LPS were able to retain *L. monocytogenes* within endosomes more effectively than unactivated resident macrophages (Table 2). These data were obtained by electron microscopic observation of macrophages, and representative micrographs are shown in Fig. 4. Proteose peptone-elicited macrophages that were not further activated *in vitro* were approximately as proficient at retaining *L. monocytogenes* within endosomes as were resident macrophages that had been activated with IFN-γ *in vitro* (Table 2). Treatment of proteose peptone-elicited macrophages with IFN-γ plus LPS enhanced their ability to retain *L. monocytogenes* within endosomes, but IFN-γ alone did not significantly increase the number of bacteria retained in endosomes.

DISCUSSION

Our results confirm previous reports that proteose peptone-elicited macrophages from several mouse strains are able to kill *L. monocytogenes* [3,4]. Exposure of such macrophages to IFN-γ for 20 h *in vitro* activated them, as indicated by increased H_2O_2 or nitrite production. However, phagocytosis and subsequent killing of *L. monocytogenes* were not generally enhanced (Table 1, Fig. 1). The duration of the listericidal assay was sufficient to have detected enhancement of bactericidal activity [5]. This confirms previous reports that IFN-γ does not increase killing of *L. monocytogenes* [3, 4] and indicates that these findings were not due to insufficient duration of the listericidal assay, as suggested by Kagaya *et al.* [5]. An IFN-γ-induced loss of listericidal activity upon acquisition of tumouricidal activity as described by Campbell *et al.* [3] was not observed in the present study. The basis for this difference in results is not known.

Treatment of macrophages with IFN-γ plus LPS further enhanced H_2O_2 or nitrite production and induced tumouricidal activity (Figs 2 and 3). Activation with IFN-γ plus LPS also enhanced phagocytosis by approximately two-fold (Fig. 1) and caused a similar increase in the number of bacteria killed during a 6-h culture (Table 1). This strongly suggests that the increase in the number of bacteria killed by macrophages activated with IFN-γ plus LPS is due at least in part to increased phagocytosis.

Several lines of evidence imply an important role for IFN-γ in the resolution of *L. monocytogenes* infections *in vivo* [13–15], but data from this study and others [3,4] fail to provide evidence for *in vitro* induction of enhanced listericidal activity by IFN-γ. This apparent paradox might be resolved by recent studies [7,16] indicating that the essential function of IFN-γ in mediating resistance to *L. monocytogenes* is enabling macrophages to retain the bacteria in endosomes. In the present study this possibility was examined by enumerating *L. monocytogenes* in the cytoplasm and in endosomes in resident and proteose peptone-elicited macrophages subjected to IFN-γ, with or without LPS, as an activation stimulus. The results obtained with resident macrophages exhibit the same pattern noted by Portnoy *et al.* [7]. However, our data (Table 2) indicate a smaller percentage of bacteria in endosomes of both resident (47%) and IFN-γ treated macrophages (76%) than reported by Portnoy *et al.* (72% and 95%, respectively) [7]. This may be due to differences in duration of activation cultures, concentration of IFN-γ, or mouse strains used. It is interesting that Portnoy *et al.* [7] noted that some bacteria that had escaped to the cytoplasm appeared damaged. This was also noted in the present study (data not shown) and may account for the effective killing of *L. monocytogenes* in the absence of 100% retention in endosomes. The mechanism for such extra-endosomal anti-microbial activity is not known. However, it seems possible that some *L. monocytogenes* that escape from endosomes may have been retained long enough to be damaged and subsequently die in the cytoplasm. Retention of *L. monocytogenes* in endosomes has not been examined previously in proteose peptone-elicited macrophages, and it is interesting that proteose peptone-elicited macrophages retain an approximately equal percentage of *L. monocytogenes* in endosomes as IFN-γ-activated resident



(*a*) (*b*) (*c*)

Fig. 4. Retention of *Listeria monocytogenes* in endosomes. Resident peritoneal macrophages from CD-1 mice or macrophages from the same population of cells used in the experiment depicted in Fig. 1 and Table 2 were plated and activated as indicated in Fig. 1 for 20 h. Macrophages were harvested for electron microscopic evaluation 4 h after addition of *L. monocytogenes*. (a) Resident macrophage (magnification ×9145) treated with culture medium. Note the dividing bacterium free in the cytoplasm; (b) resident macrophage activated with IFN-γ plus LPS (magnification ×11 198). Note *L. monocytogenes* within endosomes; (c) proteose peptone-elicited macrophage activated with IFN-γ plus LPS (magnification ×9145). Note *L. monocytogenes* within endosomes.

macrophages (Table 2). Untreated resident macrophages from CD-1 mice are marginally listericidal or non-listericidal (data not shown) [7,17] whereas IFN-γ-treated resident macrophages [7] and proteose peptone-elicited macrophages (Table 1) are consistently listericidal. Therefore, retention of *L. monocytogenes* in endosomes correlates well with listericidal activity, supporting the conclusion that this is an important mechanism in control of *L. monocytogenes* by macrophages [17]. The mechanism by which proteose peptone induces enhanced retention of *L. monocytogenes* in endosomes was not investigated, but it is possible that the host response to proteose peptone could lead to sufficient IFN-γ production *in vivo* to account for the observed increase in retention.

In vitro, IFN-γ did not significantly enhance retention in endosomes (Table 2) or killing of *L. monocytogenes* (Table 1) by proteose peptone-elicited macrophages. In contrast, IFN-γ did activate such macrophages with regard to production of H_2O_2 or nitrite (Figs 2 and 3). Thus, listericidal activity of macrophages that exhibit enhanced retention of bacteria in endosomes, as compared with resident macrophages, is not further enhanced by increasing the capacity of those macrophages to produce reactive nitrogen or oxygen intermediates. These results suggest that constitutive components of lysosomes such as hydrolytic enzymes and anti-bacterial peptides [18] may be sufficient to kill *L. monocytogenes* that cannot escape into the cytoplasm.

IFN-γ plus LPS enhances killing (Table 1) and retention of *L. monocytogenes* in endosomes (Table 2) in proteose peptone-elicited macrophages. However, the role of enhanced retention in endosomes in increased killing is unclear, because the larger number of *L. monocytogenes* killed could be accounted for by enhanced phagocytosis (Fig. 1). However, it is also possible that increased retention in endosomes is required for efficient elimination of the larger numbers of the *L. monocytogenes* phagocytosed by macrophages treated with IFN-γ plus LPS.

The data presented here indicate that IFN-γ can enhance the capacity of proteose peptone-elicited macrophages to produce reactive oxygen and nitrogen intermediates, but not their ability to kill *L. monocytogenes*. IFN-γ plus LPS increase the ability of proteose peptone-elicited macrophages to kill *L. monocytogenes*, but this may be mostly attributable to enhanced phagocytosis. Finally, proteose peptone-elicited macrophages are more capable than resident macrophages of retaining *L. monocytogenes* in endosomes. By most measures (ability to kill tumour cells or produce H_2O_2 or nitrite) these cells would not seem to be activated macrophages. However, it is apparent that enhanced retention of *L. monocytogenes* in endosomes can be induced by a stimulus (proteose peptone, *in vivo*) that is insufficient to enhance other parameters that are routinely used to assess macrophage activation. Such sensitivity to mild activation stimuli may be biologically important in host resistance to *L. monocytogenes*.

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REFERENCES

1 Adams DO, Hamilton TA. The cell biology of macrophage activation. Annu Rev Immunol 1984; **2**:283–318.

2 Adams DO, Hamilton TA. Molecular transduction mechanisms by which IFN-γ and other signals regulate macrophage development. Immunol Rev 1987; **97**:5–27.

3 Campbell PA, Canono BP, Cook JL. Mouse macrophages stimulated by gamma-interferon to kill tumor cells are not bactericidal for the intracellular bacterium *Listeria monocytogenes*. Infect Immun 1988; **56**:1371–5.

4 van Dissel JT, Strikkelbroek JJM, Michel BC, van der Barselaar MT, Leijh PCJ, van Furth R. Inability of recombinant Interferon-gamma to activate the antibacterial activity of mouse peritoneal macrophages against *Listeria monocytogenes* and *Salmonella typhimurium*. J Immunol 1987; **139**:1673–8.

5 Kagaya K, Watanabe K, Fukazawa Y. Capacity of recombinant gamma-interferon to activate macrophages for Salmonella-killing activity. Infect Immun 1988; **57**:609–15.

6 Tran TTH, Cook AD, Ganas M, Cheers C. Distinction between "inflammatory" and "immune" macrophages killing *Listeria monocytogenes* in murine infection. Immunol Cell Biol 1990; **68**:289–97.

7 Portnoy DA, Schreiber RD, Connelly P, Tinley LT. γ-Interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. J Exp Med 1989; **170**:2141–7.

8 Pick E, Mizel D. Rapid microassay for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J Immunol Methods 1981; **46**:211–26.

9 Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol 1988; **141**:2407–12.

10 Friedman A, Beller DJ. Simultaneous expression of Ia and cytocidal activity by macrophages, and the consequences for antigen presentation. Immunology 1987; **61**:435–41.

11 Zar JH. Biostatistical analysis. 2nd edn. Englewood Cliffs, NJ: Prentice-Hall, 1984.

12 Cooper PH, Mayer P, Daggiolini M. Stimulation of phagocytosis in bone marrow derived mouse macrophages by bacterial lipopolysaccharide: correlations of biochemical and functional parameters. J Immunol 1984; **133**:913–22.

13 Buchmeier NA, Schreiber RD. Requirement of endogenous interferon-γ production for resolution of *Listeria monocytogenes* infection. Proc Natl Acad Sci USA 1985; **82**:7404–8.

14 Cheers C, McKenzie IFC, Pavlov H, Wade C, York J. Resistance and susceptibility to bacterial infection: course of listeriosis in resistant or susceptible mice. Infect Immun 1987; **19**:763–70.

15 Kinderlen AF, Kaufmann SHE, Lohmann-Matthes ML. Protection of mice against the intracellular bacterium *Listeria monocytogenes* by recombinant immune interferon. Eur J Immunol 1984; **14**:964–7.

16 Portnoy DA, Jacks PS, Hinrichs DJ. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J Exp Med 1988; **167**:1459–71.

17 Bielecki J, Youngman P, Connelly P, Portnoy DA. *Bacillus subtilis* expressing a haemolysis gene from *Listeria monocytogenes* can grow in mammalian cells. Nature 1990; **345**:175–6.

18 Boman HG. Antibacterial peptides: key components needed in immunity. Cell 1991; **65**:205–7.