HOST RESPONSE TO INFECTION

Killing mechanism of *Listeria monocytogenes* in activated macrophages as determined by an improved assay system

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Exposure of *Listeria monocytogenes* to gentamicin 5 mg/L for 4 h resulted in the killing of most extracellular bacteria, but had no effect on the survival of bacteria inside macrophages. Higher concentrations of gentamicin caused a reduction in the number of intracellular bacteria. This effect was associated with cellular uptake of gentamicin, but was unaffected by activation of macrophages by interferon- γ and lipopolysaccharide. In experiments in which exposure to gentamicin 5 mg/L for 4 h was used to kill extracellular bacteria, killing by activated macrophages was impaired when O_2^- production was inhibited by superoxide dismutase, but not when nitric oxide production was blocked by N^G -monomethyl-L-arginine. These data suggest that the reactive oxygen intermediates are more important than nitric oxide in the killing of *L. monocytogenes*, at least in macrophages activated *in vitro*.

Introduction

Listeria monocytogenes is a facultative intracellular pathogen capable of survival and proliferation in macrophages and epithelial cells [1–3]. Entry of *L. monocytogenes* into cells is mediated by the *inlAB* and *iap* genes encoding internalin and protein p60, respectively [4, 5]. Soon after internalisation, the bacterium escapes from the endosomal compartments into the cytoplasm by means of the production of listeriolysin O which is a major virulence factor [6, 7]. Although *L. monocytogenes* can survive in macrophages of normal mice, bacteria are completely eliminated from infected organs after the establishment of protective immunity [8–10].

It is generally believed that protective immunity depends on the enhancement of intracellular killing of macrophages activated by various cytokines, especially interferon- γ (IFN- γ). When macrophages are stimulated with macrophage-activating factor *in vitro*, the production of reactive oxygen intermediates is enhanced [11, 12], nitric oxide is induced [13] and phagosome-lysosome fusion is facilitated [14]. To investigate the killing of bacteria by macrophages *in vitro*, antibiotics are used to kill residual extracellular bacteria, and viable

intracellular bacteria are counted at various times during the assay. Gentamicin has often been used to remove extracellular bacteria in such assays, but there is evidence that this antibiotic may affect the viability or growth of intracellular bacteria [15].

The present study investigated the influence of various concentrations of gentamicin on the growth of *L. monocytogenes* inside and outside macrophages. The system developed was then used to examine the role of reactive oxygen intermediates and nitric oxide in the killing of *L. monocytogenes* in macrophages activated by IFN- γ and lipopolysaccharide (LPS).

Materials and methods

Bacteria

A laboratory stock culture of *L. monocytogenes* EGD, a virulent strain, was used throughout. The bacteria were grown in Tryptic Soy Broth (Difco) at 37°C for 16 h, washed repeatedly, suspended in phosphate-buffered saline and stored at –70°C until use.

Reagents

Superoxide dismutase and N^G -monomethyl-L-arginine (NMMA) were purchased from Wako Pure Chemical Inc. (Osaka, Japan). Recombinant murine IFN- γ was provided by the Central Research Institute, Daiichi

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Seiyaku Co. Ltd (Tokyo, Japan). LPS (*Escherichia coli* O111:B4) was purchased from Difco. Gentamicin reagent solution was purchased from Life Technologies Inc. (Grand Island, NY, USA).

Cell-free culture of bacteria

L. monocytogenes was cultured in RPMI 1640 (Flow Laboratories Inc., Maclean, VA, USA) supplemented with various concentrations of gentamicin for 8 h. Every 2 h, the number of survivors (cfu/ml) was assessed by subcultivation on Brain Heart Infusion (BHI) Agar (Eiken Chemical Co. Ltd, Tokyo, Japan).

Preparation of macrophages

Male mice (7–10 weeks old) of ICR strain (Charles River Japan, Atsugi, Japan), raised and maintained under specific pathogen-free conditions, were used. Peritoneal exudate cells were obtained from the mice 3 days after intraperitoneal injection of 2 ml of Thioglycollate Medium (Difco) 3%. The cells were washed with Hanks's Balanced Salts Solution (HBSS) and suspended in the medium consisting of RPMI 1640 supplemented with heat-inactivated fetal bovine serum 10%, gentamicin 10 mg/L, HEPES 5 g/L and NaHCO_3 2 g/L. Peritoneal exudate cells (2×10^6) were cultured in a 24-well flat-bottomed tissue culture plate (Coster, Cambridge, MA, USA) for 2 h at 37°C; non-adherent cells were removed by gentle washings with warm HBSS, and the culture medium in each well was replaced with 1 ml of fresh medium. Adherent cells thus prepared were used as macrophages. The cells were stimulated with murine rIFN- γ (100 U/ml) and LPS (10 mg/L) for 20 h before use in the intracellular killing assay. After culture for 20 h the culture medium was replaced with antibiotic-free medium.

Intracellular killing assay

The procedure is shown in Fig. 1. Macrophages were infected with *L. monocytogenes* at a ratio of 5:1 bacteria:cell. The plates were centrifuged at 450 *g* for 5 min to enhance the attachment of bacteria to macrophages and incubated at 37°C for 60 min to facilitate the ingestion of bacteria. Extracellular bacteria were removed by washing seven times with 1 ml of warm HBSS, and gentamicin was then added to the culture medium to achieve a concentration of 5 mg/L unless otherwise stated. Four hours later (0 time), counts (cfu) of viable intracellular bacteria were estimated by serial dilution and plating on BHI agar after disruption of the cells with sterile distilled water to release the intracellular bacteria. After a further 2 h, the number of viable bacteria inside cells was counted again and the difference was regarded as the level of macrophage killing activity. The number of viable bacteria remaining extracellularly was similarly estimated by subculture of washed (20 times in HBSS) survivors in supernates of the macrophage assay plates.

Fig. 1. Experimental procedure for the investigation of intracellular killing of *L. monocytogenes*. MOI, multiplicity of infection; SOD, superoxide dismutase.

Nitrite determination

Nitrite concentration in the macrophage culture was used as a measurement of nitric oxide synthesis. It was assayed by a standard Griess reaction adapted to microplates as described previously [16]. A 100- μ l volume of Griess reagent (equal volumes of sulphanilamide, 1.5% in H_3PO_4 5% and naphthylethylene diamine dihydrochloride, 0.1% in H_2O) was mixed with an equal volume of test sample (culture supernate) and incubated at room temperature for 10 min. Absorbance of the chromophore formed was measured at 540 nm in an automated microplate reader. Nitrite was quantified with NaNO_2 as a standard and the results were expressed as μM of nitrite.

Accumulation of gentamicin inside macrophages

Peritoneal macrophages were exposed to various concentrations of gentamicin during stimulation with IFN- γ and LPS as described above. The cells were harvested and centrifuged. The culture medium was discarded, then the cells were resuspended in 2 ml of HBSS and transferred to a new tube for further washing. The cells were washed twice more with 2 ml of HBSS, and the cell lysate was prepared by ultrasonication for assay of gentamicin. Gentamicin concentration was determined by reverse-phase high-performance liquid chromatography (HPLC) as follows: an L-column ODS (4.6×150 mm, 5 μm particle size; Chemicals Inspection and Testing Institute, Tokyo, Japan) maintained at 40°C was used as the analytical column. The mobile phase (0.65 M NaClO_4 , 0.04 M NaH_2PO_4 , 0.007 M *m*-xylenesulphonic acid sodium salt and acetonitrile 4.3%) was used at a flow rate of 1.5 ml/min. The injection volume was 100 μ l, and fluorescence intensity produced by the reaction of *o*-

phthalaldehyde (pH 10.5) was measured with excitation set at 340 nm and emission at 435 nm. The protein concentration of cell lysate was calculated according to the absorbance at 280 nm and 260 nm.

Results

Effect of gentamicin on the killing of *L. monocytogenes*

In cell-free culture, gentamicin at concentrations above 3 mg/L killed 99% of the bacteria within 4 h (Fig. 2). In cell culture, gentamicin 5 mg/L had no influence on

the number of bacteria inside macrophages, whereas the number of viable extracellular bacteria was reduced considerably (Fig. 3). After pre-incubation of infected macrophages with gentamicin 5 mg/L for 4 h, subsequent exposure of the washed cells to gentamicin at concentrations ≤ 5 mg/L had little effect on the growth of *L. monocytogenes* inside macrophages, but concentrations ≥ 10 mg/L reduced intracellular survival (Fig. 4). Assay of gentamicin in cell lysates showed that the intracellular level of gentamicin was increased in a dose-dependent way when the extracellular level was increased (Fig. 5). There was no significant difference in the gentamicin levels inside non-activated macrophages and those activated with IFN- γ and LPS (Fig. 5).

Fig. 2. Effect of gentamicin on the survival of *L. monocytogenes* in a cell-free medium (mean and SD of three experiments). ×, 0.2 mg/L; ◇, 0.39 mg/L; □, 0.78 mg/L; △, 1.56 mg/L; ○, 3.125 mg/L; ■, 6.25 mg/L; ▲, 12.5 mg/L; ●, 25 mg/L.

Fig. 4. Effect of exposure for 2 h to various concentrations of gentamicin on intracellular killing of *L. monocytogenes* after pre-incubation with gentamicin 5 mg/L for 4 h (mean and SD of three experiments).

Fig. 3. Counts of ■ intracellular and ▨ extracellular bacteria before and after exposure of infected peritoneal macrophages to gentamicin 5 mg/L for 4 h (mean and SD of three experiments).

Fig. 5. Intracellular gentamicin concentrations in ▨ normal and ■ activated macrophages as measured by reverse-phase HPLC of cell lysates, after exposure of cells to gentamicin at the concentrations shown.

Effect of superoxide dismutase and NMMA on the intracellular survival of *L. monocytogenes* in activated macrophages

Stimulation of macrophages with IFN- γ and LPS resulted in the enhancement of listericidal activity in the present assay system, but the listericidal activity of activated macrophages was impaired after addition of superoxide dismutase (Fig. 6). When macrophages were treated with IFN- γ and LPS, a large amount of nitrite was produced, but after the addition of NMMA, nitrite production was inhibited (data not shown). However, NMMA did not impair the listericidal activity of macrophages stimulated with IFN- γ and LPS (Fig. 7).

Discussion

Macrophages are the most important effector cells in the defence of the host against infection by intracellular bacteria including *L. monocytogenes*. A reliable assay system for the intracellular killing activity of macrophages is of critical importance, especially for the understanding of mechanisms for the expression of protective immunity. One of the key requirements for the accuracy of the in-vitro bactericidal assay is to eliminate the bacteria remaining extracellularly after ingestion by macrophages. For this purpose, gentamicin at a concentration of *c.* 50 mg/L has been widely used, as this antibiotic is believed to be incapable of penetrating the macrophage membrane. However, there are reports that such a high concentration of gentamicin penetrates HeLa cells, reaching *c.* 90% of the extracellular concentration after incubation for 72 h

Fig. 6. Effect of superoxide dismutase (SOD) on the intracellular growth of *L. monocytogenes* in macrophages pretreated with IFN- γ and LPS (mean and SD of three experiments).

Fig. 7. Effect of NMMA on the intracellular growth of *L. monocytogenes* in macrophages pretreated with IFN- γ and LPS (mean and SD of three experiments).

[17]. Drevets *et al.* [15] also showed that extracellular gentamicin, used at 50–100 mg/L, could accumulate within macrophages and pointed out that the use of gentamicin in macrophage bactericidal assays might result in an incorrect interpretation of experimental results.

The present study tried to establish a reliable assay system with special reference to the concentration of gentamicin that effectively kills extracellular bacteria without affecting growth of intracellular *L. monocytogenes*. The results (Figs. 3 and 4) suggest that 5 mg/L of gentamicin is a suitable concentration to use in the intracellular bactericidal assay. However, this concentration may affect intracellular *L. monocytogenes* on prolonged incubation for *c.* 24 h [18]. Accordingly, when a longer observation time is required, the cells should be cultured in gentamicin at a concentration < 5 mg/L after eliminating extracellular bacteria. The use of a higher concentration of gentamicin resulted in the accumulation of antibiotic inside cells (Fig. 5).

Incubation of macrophages in the presence of recombinant IFN- γ and LPS resulted in enhanced intracellular killing of *L. monocytogenes* in the present assay system. Measurement of intracellular gentamicin revealed that this was not due to an enhanced uptake of antibiotic as postulated by Drevets *et al.* [15]. Macrophages stimulated with IFN- γ and LPS exhibit an enhanced generation of O_2^- . In the present system, when O_2^- production was inhibited by adding superoxide dismutase, the killing ability of activated macrophages was impaired (Fig. 6). The results confirmed that O_2^- was involved in the killing of *L.*

monocytogenes by activated macrophages and confirmed the reliability of the present assay system.

Nitric oxide is a toxic radical synthesised from L-arginine by inducible nitric oxide synthase which is expressed mainly in activated cells of macrophage lineage [19]. IFN- γ and LPS are potent inducers of nitric oxide synthase in macrophages [19]. Nitric oxide exhibits antimicrobial activity *in vitro* against various pathogens, such as *Leishmania major* [20], *Toxoplasma gondii* [21] and *Mycobacterium bovis* [22], and may be a critical effector molecule in the killing of *L. monocytogenes* by IFN- γ -activated macrophages [23], although reports are conflicting [24–26]. In the present study, when peritoneal macrophages were stimulated with IFN- γ and LPS, a large amount of nitric oxide was observed as determined by nitrite in the supernates of the culture (data not shown). Surprisingly, there was no change in the killing function of activated macrophages even in the presence of NMMA, which completely inhibited the production of nitrite (Fig. 7). These data do not support a primary role for nitric oxide in the killing of intracellular *L. monocytogenes* in macrophages pre-activated by IFN- γ and LPS *in vitro*. The results suggest that the reactive oxygen radicals were more important than nitric oxide in the killing of *L. monocytogenes* in these conditions.

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