BACTERIAL PATHOGENICITY

Opsonin-independent adherence and phagocytosis of *Listeria monocytogenes* by murine peritoneal macrophages

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***Listeria monocytogenes* adhered to and multiplied intracellularly in murine peritoneal macrophages in the absence of opsonins. The infective process in these cells was evaluated by viable bacterial cell colony counts of intracellular organisms and documented by transmission and scanning electron microscopy. Adherence of listeriae to macrophages involved surface interactions of the prokaryotic cell surface and eukaryotic cell membranes. Subsequent phagocytosis was seen to occur through a process in which host cell-derived pseudopodia surrounded and engulfed organisms leaving them within phagosomes in the cytoplasm of infected cells. This process of uptake of *L. monocytogenes* by macrophages occurred at 4°C. Following invasion of the cell, escape of *L. monocytogenes* from the phagosome into the cytoplasm was initiated as early as 10 min into the infective process. Intracellular multiplication of bacteria continued for 8 h after inoculation at which point loss of adherent macrophages due to cell lysis was evident. The mean generation time of the organism in these cells was 58 min. The cellular and ultrastructural events of *L. monocytogenes* adherence to and phagocytosis by murine macrophages in the absence of antibody or complement have been defined.**

Introduction

Listeria monocytogenes is the causal agent of listeriosis, the clinical manifestations of which range from mild influenza-like symptoms to meningoencephalitis and septic abortion. The disease primarily affects those individuals who are immunocompromised, the very young and the elderly, as well as transplant recipients and pregnant women.

L. monocytogenes is a facultative intracellular bacterial pathogen which serves as a useful model for the study of intracellular infections in general, as well as to investigate the cell-mediated immune response and the processes of inflammation in the host [1]. Establishment of intracellular infection is brought about by an initial recognition or attachment event between binding molecules or adhesins on the organism and receptors located at the host cell membrane surfaces. The process eventually leads to internalisation of the pathogen by infected cells. In experimental murine infections, *Listeria* organisms were shown to accumulate primarily in the liver and spleen, replicating within mononuclear phagocytic cells as well as in cells that are not professionally phagocytic including hepatocytes [2, 3]. Intracellular growth of the organism

in host cells has been characterised in the transformed human enterocyte-like cell line Caco-2 [4] and macrophage-like J774 cells [5].

Attachment and entry of the bacteria occurs through their apical surfaces and these processes do not appear to be actin-dependent [6]. After adherence to host cells, bacteria are taken into membrane-bound phagosomes. Subsequently, the organism escapes from the phagosome and enters the cytoplasm, where the bacteria replicate [7]. Following escape from the phagocytic vacuole, the organism becomes coated with actin filaments to form a large tail or comet-like structure up to 5 µm long [8]. This organism-actin complex is critical for the intercellular spread of *L. monocytogenes* in that as the actin polymerises, the pathogen is 'pushed' to the surface of the host cell and a cytoplasmic pseudopod projection is produced with the listeria at the tip. This pseudopod touches a neighbouring cell which phagocytoses it, enclosing the organism in a double-membrane vacuole [8–10]. The organism then escapes from the vacuole and repeats this cycle to initiate further infection of neighbouring cells. In this fashion, organisms remain cell-associated throughout the infective process and thereby avoid direct attack by the humoral immune system of the host [7].

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Mounting a successful host defence against this

pathogen involves a patent cell-mediated immune response with macrophage killing of internalised bacteria [1]. However, it has been shown that mononuclear phagocytes are heterogeneous with regard to listericidal activity. Murine peritoneal macrophages produced by stimulation with thioglycollate permit intracellular growth of *L. monocytogenes*, whereas macrophages stimulated by proteose-peptone are listericidal [11]. Furthermore, these workers showed that thioglycollate-stimulated macrophages utilised complement receptor 3 (CR3) as a minor binding molecule for listeriae. This suggested that an alternative host cell receptor was responsible for binding the majority of organisms to macrophages in non-listericidal fashion. In the present study, the binding and uptake of *L. monocytogenes* to host cells in the absence of opsonins was investigated. Bacterial uptake in an opsonin-deficient environment may occur at the very earliest stages of disease and before the induction of antibody or increased levels of complement. Such opsonin-independent mechanisms may offer intracellular survival advantages for *Listeria* spp.

Materials and methods

Organisms and culture conditions

A fully virulent clinical isolate of *L. monocytogenes* serotype 1/2b was isolated on blood agar, subcultured once on Tryptic soy agar, and stored as stock cultures frozen at -70°C in serum-sorbitol 1%. Virulence was assayed with fertile hens' eggs [12] and over the course of this study the LD₅₀ for this isolate remained unchanged at 23 cfu. Thawed samples were plated on agar and incubated overnight. Colonies were harvested, resuspended in Tryptic soy broth, and cultured for 8.5 h to mid-exponential growth phase with shaking at 37°C . Cells were collected by centrifugation at 5000 *g*, washed with Hanks's Balanced Salts Solution (HBSS; Sigma) and resuspended in HBSS to give 1×10^9 cfu/ml prior to inoculation of macrophages.

Murine peritoneal macrophages

BALB/c mice of both sexes were used at 3–6 months of age. Murine peritoneal macrophages were elicited by intraperitoneal injection of 1.5 ml of thioglycollate per mouse. Mice were killed by CO₂ asphyxiation 2.5–3.5 days later, and peritoneal exudate cells were extracted by peritoneal cavity lavage with three 10-ml volumes of HBSS. These cell extracts were pooled and the cells were collected by centrifugation at 220 *g* for 10 min. Red blood cells were lysed by the addition of 9 ml of sterile distilled water followed by 1 ml of $10 \times$ HBSS. The cells were then washed twice with HBSS and the total number was determined by haemocytometer.

Transmission electron microscopy

Murine peritoneal macrophages at a concentration of

1×10^7 cells/ml and bacterial suspensions were held separately on ice for 10 min before mixing. Bacteria were added to suspensions of macrophages at a multiplicity of infection (MOI) of 100 bacteria/host cell in 1.5 ml of HBSS. The inoculated cell preparations were centrifuged at 220 *g* for 10 min to pellet the macrophages and then at 850 *g* for 10 min to deposit the bacteria on to the cells [13]. During these manipulations the suspensions of bacteria and macrophages were maintained at 4°C with the aim of inhibiting phagocytosis. The tubes were then warmed immediately by gentle rotation in a water bath at 37°C to facilitate phagocytosis. Samples were collected after 1, 2, 3, 3.5, 5, 10, 15, 20, 30, 40 and 60 min intervals by centrifugation at 4°C . For each time point, a sample was placed on ice and the supernate was removed. The harvested pellets were fixed with prewarmed glutaraldehyde 5% v/v in 0.05 M cacodylate buffer containing 10 mM MgSO₄ at pH 7.2 (CB). A 0 min sample was collected immediately after the initial centrifugation steps and this was fixed in glutaraldehyde in CB pre-cooled to 4°C .

Samples were washed 10 times with CB, and pre-embedded in Noble agar 2% w/v at 55°C . Cells in agar were cut into 1-mm³ blocks and post-fixed in OsO₄ 1% w/v in CB for 36 h. Samples were then dehydrated in an ethanol series to propylene oxide and embedded in an epon-araldite resin mixture. After polymerisation at 60°C for 24 h, blocks were trimmed and sectioned on an LKB ultramicrotome III. Sections of *c.* 60 nm thickness were stained for 1 min each with uranyl acetate 5% w/v and lead citrate 0.4% w/v, and examined at 75 kV in the transmission mode of a H-600 scanning-transmission electron microscope.

Scanning electron microscopy

For scanning electron microscopy (SEM), macrophages were harvested as described above. Three ml of RPMI-1640 medium, which contained 1×10^6 cells in fetal bovine serum (FBS) 10% were seeded into wells of six-well culture plates (Costar, Cambridge, MA, USA), each of which contained a sterile 22-mm diameter glass coverslip. The cells were incubated at 37°C for 3 h. The cultures were washed three times with HBSS to remove unbound cells and cell culture medium. Two ml of HBSS were placed on the cells and *L. monocytogenes* was added in HBSS at a MOI of 100 bacteria/host cell (1×10^8 cfu/well). The plates were then incubated at 37°C in the presence of CO₂ 5% and samples were taken at prescribed times points for 1 h. Non-adherent bacteria were removed by washing the cell cultures three times with HBSS and the cells were fixed in glutaraldehyde 5% v/v in CB for 24 h. Samples were then washed 10 times with CB *in situ*, and dehydrated with a graded ethanol series followed by drying from hexamethyldisilizane (Electron Microscopy Sciences, Fort Washington, PA, USA). Monolayers were then sputter coated with 20 nm of gold-palladium in a

Hummer V sputter coater and observed in an AMR 1000 scanning electron microscope at 60 kV.

Intracellular growth studies

To define the binding, uptake and intracellular replication of *L. monocytogenes* in peritoneal macrophages further, viable bacterial colony counts (VBCC) were used. Macrophages were placed in six-well plates at 1×10^6 cells/well and allowed to adhere for 8 h. Cultures were washed three times with HBSS to remove non-adherent cells and serum. *L. monocytogenes* cells were suspended in HBSS and added to the wells at a MOI of 1. Bacteria were allowed to adhere to and penetrate macrophages for 1 h at 37°C. The cells were washed three times to remove non-adherent bacteria and RPMI-1640 with FBS 10% and gentamicin 6 µg/ml was added to the cell monolayers. After washing to remove antibiotic, samples were collected each hour for 9 h (Fig. 1) by which time an appreciable loss of adherent macrophages was noted. Adherent cells were lysed in 2 ml of sterile water and disrupted by agitation with a Pasteur pipette. For each time period, a 100-µl sample was removed from the lysate samples and serially diluted in peptone 1% and plated in duplicate on Tryptic soy agar for enumeration of intracellular listeriae. Results were expressed as cfu/ml of macrophage lysate.

Results and discussion

Successful adherence to and infection of murine peritoneal macrophages by *L. monocytogenes* occurred

Fig. 1. Intracellular growth of *L. monocytogenes* in murine peritoneal macrophages; c. 14% of the bacterial inoculum adhered to macrophages. Mean generation time between 3 and 8 h was 58 min. Arrow indicates time at which gentamicin was added. *Number of organisms attached to macrophages at time of addition of gentamicin. Cell lysis was evident by 8 h of intracellular growth.

in the absence of the opsonic components, complement or antibody. SEM observations of monolayers showed numerous listeriae adherent to filopodia extending from macrophages (Fig. 2A) as well as organisms bound directly to the surface membranes of these cells (Fig. 2B). In both cases there were few host cell changes. Extracellular bacteria apparently bound to the macrophages in all stages of infection involved single bacteria or organisms in the process of binary fission. Observation of TEM thin-sections showed electron-diffuse cytoplasmic material streaming from apparent lytic points in the host cell surface membrane in apposition to organisms (Fig. 2C and inset). This phenomenon was undoubtedly due to the localised action of listeriolysin O produced by individual organisms. Indeed, the presence of ≥ 10 listeriae bound to individual macrophages frequently induced cell lysis in the absence of infection. This destruction detected by electron microscopy was confirmed during growth studies of macrophages infected with *L. monocytogenes* at a MOI of 100. Under conditions of high inocula, host cells were lost rapidly due to lysis and by 4 h after inoculation, >90% of the macrophages were non-adherent. Heavily infected Caco-2 cells have also been reported to lyse over a similar period [14].

Examination of peritoneal macrophages by TEM showed listeriae within cells in all samples, including those cells taken immediately after centrifugation. Large numbers of the cell-associated listeriae were located within the cytoplasm of macrophages despite being maintained on ice, which indicated that uptake of the organism in the absence of opsonins occurred at low temperatures. Similar findings have been reported for an opsonin-mediated system [15]. At the earliest stages of infection of macrophages, all intracellular bacteria were present in membrane-bound phagosomes. The first bacteria seen partially free in the cytoplasm of peritoneal macrophages occurred in samples collected 10 min after infection (Fig. 2D). This phenomenon was detected earlier in these primary macrophages than was reported for the transformed macrophage-like cell line, J774, in which free listeriae were seen in the cytoplasm 30 min after inoculation [8]. *L. monocytogenes* was taken up by the usual mechanisms of phagocytosis (Fig. 2E) in the absence of coiling phagosomes [13]. This may be due to the absence of opsonic components in this system. Indeed, coiling phagosomes were not observed during initial infection of U-937 cells with *L. pneumophila* in the absence of opsonins [16]. The molecular events that mediate uptake may vary in the absence or presence of opsonins; however, these differences remain to be established. As early as 1 min after inoculation, numerous intracellular bacteria were seen within phagosomes by TEM (Fig. 2F).

It has been postulated that the receptor by which intracellular pathogens are recognised by macrophages may define their intracellular fate [17]. In a study

Fig. 2. Ultrastructure of the adherence and opsonin-independent uptake of *L. monocytogenes* by murine peritoneal macrophages. **A**, Scanning electron micrograph of a macrophage with multiple organisms shown bound to host cell filopodia; $\times 4500$; bar = $2\ \mu\text{m}$. **B**, Scanning electron micrograph of a macrophage with numerous listeriae bound directly to the cytoplasmic membrane; $\times 3600$; bar = $2\ \mu\text{m}$. **C**, Transmission electron micrograph of a macrophage with a single bacterium undergoing wash-resistant attachment at 5 min after inoculation; $\times 22\ 950$; bar = $0.5\ \mu\text{m}$. Inset: Close association of organism surface with host cell plasma membrane is evident. Note macrophage cytoplasmic material between organism and macrophage membrane (arrow) released due to the localised action of listeriolysin O; $\times 93\ 600$; bar = $0.1\ \mu\text{m}$. **D**, Internalised organism showing partial escape of the bacterium from the phagosome occurring at 10 min after infection; $\times 17\ 550$; bar = $0.5\ \mu\text{m}$. **E**, Transmission electron micrograph of organisms undergoing phagocytosis 3 min after inoculation. Note pseudopodia engulfing organisms; $\times 9\ 360$; bar = $1\ \mu\text{m}$. **F**, Transmission electron micrograph of macrophage showing many internalised listeriae at 1 min after inoculation; $\times 11\ 700$; bar = $1\ \mu\text{m}$.

outlining the uptake of *Mycobacterium tuberculosis* it was shown that when bacterial entry was mediated by Fc receptors, lysosome-associated phagocytic vesicles resulted, whereas entry via CR3 receptors induced the formation of lysosome-free vesicles [17]. It is possible that such differences exist in the intracellular fate of *L. monocytogenes* taken up by opsonin-dependent and opsonin-independent mechanisms.

VBCC count data showed that *c.* 2.3×10^5 listeria attached to 1×10^6 macrophages by 1 h after inoculation, indicating that *c.* 14% of the bacterial inoculum had bound to the cell monolayers. Growth curves of *L. monocytogenes* demonstrated that the organism did not replicate in unsupplemented HBSS. The minimum inhibitory concentration (MIC) of gentamicin in RPMI-1640 with FBS 10% was determined as $0.6 \mu\text{g/ml}$ for this strain. By 2 h after the addition of gentamicin at 10-fold the MIC, the number of cfu measured declined to 7% of the originally adherent bacteria, indicating death of extracellular listeriae. The mean generation time (Fig. 1) in these macrophages was shown to be *c.* 58 min, compared to 90 min reported for Caco-2 cells [14] and 40 min seen in bone-marrow-derived murine macrophages [7]. At 9 h after inoculation, the monolayers were significantly disrupted by the infective process and >95% of the macrophages were detached or lysed.

This work demonstrated the effective adherence and uptake of *L. monocytogenes* by murine peritoneal macrophages in an opsonin-independent fashion. It has been shown that thioglycollate-elicited, non-listericidal macrophages take up *Listeria* cells through CR3 only 33% of the time [15]. In addition, non-listericidal macrophages phagocytosed listeria more efficiently than did proteose-peptone-elicited, listericidal macrophages. In contrast, CR3 mediated *c.* 66% of the uptake of serum-opsonised listeria by listericidal macrophages and of these phagocytosed organisms only 2% were successful in escaping from the phagosome into the cytoplasm as compared to bacteria phagocytosed by non-listericidal macrophages. Furthermore, these organisms showed an increase in the number of actin-coated bacteria compared with the number of bacteria phagocytosed. Successful binding of this pathogen to macrophages facilitates replication and enhances infection of the host. Therefore, it seems certain, that these opsonin-independent binding processes play an important role in the successful infection of permissive host macrophages by *L. monocytogenes*, especially in the very early stages of listeriosis.

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