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RESEARCH ARTICLE

Studies on the susceptibility of different culture morphotypes of *Listeria monocytogenes* to uptake and survival in human polymorphonuclear leukocytes

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Abstract

This study demonstrated that atypical virulent filaments of *Listeria monocytogenes* (rough variant type II and designated FR for this study), isolated from clinical specimens or generated during exposure to pulsed-plasma gas discharge in liquids, were shown to be capable of survival when engulfed by human polymorphonuclear leukocytes (PMNLs). Factors shown to significantly influence the maximal respiratory burst response in PMNLs and survival of different internalized cell or filament forms of *L. monocytogenes* were bacterial strain, culture form, degree of opsonization (with and without the use of 10% serum) and composition of the bacterial growth media used before uptake by PMNLs. Opsonized regular-sized *L. monocytogenes* cells grown on blood agar (BA) elicited the greatest respiratory burst response and survived best in PMNLs. The filamentous (FR) and multiple cell chain (MCR) rough variants were significantly less susceptible to uptake and survival in PMNLs. Supplementation of tryptone soya agar with hemin resulted in significantly reduced chemiluminescence responses in phagocytosing PMNLs compared with the maximal levels observed from prior bacterial growth on BA or brain heart infusion agar that also contained a source of iron. The MCR variants secreting decreased levels of a peptidoglycan hydrolase CwhA protein exhibited the lowest percentage survival when internalized in PMNLs compared with wild-type smooth or FR culture variants as determined by the macrophage-killing assay.

Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, facultative intracellular bacterium that can cause severe food-borne infections in humans and animals (Vázquez-Boland *et al.*, 2001). The major risk groups are immunocompromised individuals and pregnant women. The infective dose of this organism is yet to be determined, and is most probably complicated by a number of factors, including the susceptibility of the host and the virulence of the infecting strain. Virulent strains of *L. monocytogenes* are able to survive and multiply within host macrophages, and can invade, replicate and multiply in nonprofessional phagocytes such as mouse 3T6 fibroblasts, hepatocytes and human colon carcinoma Caco-2 cells (Gaillard *et al.*, 1987; Dramsi *et al.*, 1995; Drevets *et al.*, 1995; Ohya *et al.*, 1998). Its ability to replicate in the cytoplasm of the cells of the host is critical for virulence, as evidenced by the significant attenuation of *L. monocytogenes* when intracellular replication is compromised by mutation (Hardy *et al.*, 2004).

Changes in bacterial colony morphology often accompany microbial adaptation to new environments and ecological niches (Medina *et al.*, 2003; Monk *et al.*, 2004). *Listeria monocytogenes* has also been reported to form atypical elongated (filamentous) cells when exposed to a range of adverse growth conditions, such as high concentrations of NaCl (8–9%) in the presence (Bereski *et al.*, 2002) or absence of acid (Brezin, 1973; Isom *et al.*, 1995; Jørgensen *et al.*, 1995; Bereski *et al.*, 2002), acidic conditions, i.e. pH 5.0 at 37 °C (Isom *et al.*, 1995), increased CO_2 environments (Nilsson *et al.*, 2000; Lie *et al.*, 2003), high hydrostatic pressure (Ritz *et al.*, 2001), osmotic stress (Jørgensen *et al.*,

1995), alkaline stress (Giotis *et al.*, 2007), above optimum growth temperature, i.e. 42.5 °C (Rowan & Anderson, 1998), and the presence of antimicrobial growth agents such as trimethorpim and cotrimoxazole (Minkowski *et al.*, 2001). A number of studies suggest that, in some cases, removal of such deleterious stresses results in a slow return to normal wild-type cell forms within 24 h (Brezin, 1973; Isom *et al.*, 1995), suggesting that filamentation of *L. monocytogenes* may be an adaptive response to adverse growth conditions. Monk *et al.* (2004) had previously observed that chaining cells exhibited enhanced biofilm-forming capabilities. The colonization of the murine gall bladder by *L. monocytogenes* also resulted in a change in cellular morphology (chaining), subsequently leading to the formation of biofilms within the gall bladder, which resembles a dynamic rather than a static environment (Hardy *et al.*, 2006). Variant rough colony morphotypes were thought to occur spontaneously and irreversibly at a low frequency during prolonged culture in the laboratory. Apart from obvious physical differences, such as the absence of a blue-green sheen upon Henri oblique illumination and impaired cell separation that produced chaining cells without coordinated motility, the fermentative and biochemical profiles of rough and smooth colonies were considered identical (Gutekunst *et al.*, 1992; Rowan *et al.*, 2000; Monk *et al.*, 2004).

Characterization of the molecular determinants involved in conversion to the filamentous FR colony variants has been previously described by a number of researchers (Kuhn & Goebel, 1989; Gutekunst *et al.*, 1992; Monk *et al.*, 2004), with the specific involvement of a peptidoglycan hydrolase CwhA (formerly termed invasion-associated protein or p60; Wuenscher *et al.*, 1993) in the formation of the rough colony morphotype. These so-called type 1 rough colony isolates showed reduced CwhA secretion plus decreased attachment and invasion of certain nonphagocytic cell lines (Gutekunst *et al.*, 1992), while a CwhA null mutant was previously used to clarify the role of the CwhA protein during infection (Pilgrim *et al.*, 2003). The isolation of a filamentous rough colony variant (termed type II) showing wild-type levels of CwhA secretion and cellular invasion has also been described previously (Rowan *et al.*, 2000; Lenz & Portnoy, 2002). While the specific mechanisms governing these morphological changes still remain unclear (Gardan *et al.*, 2003; Monk *et al.*, 2004; Giotis *et al.*, 2007), conversion to the atypical rough culture forms may result in a failure to accurately detect and/or enumerate this enteropathogen from adverse conditions such as food preservation or inimical stresses associated with exposure to the host immune system. Although FR variants have been isolated previously from clinical specimens and food samples (Rowan *et al.*, 2000), the multiple long cell chain form (MCR type) secreting reduced levels of CwhA has not. Despite a plethora of published data on the impact of food-processing stresses and associated similarities to deleterious stresses encountered by bacterial pathogens on exposure to host immune cells on the survival and morphological stability of *L. monocytogenes*, very little information currently exists on the susceptibility and/or the survival of different culture morphotypes of this pathogen to a phagocytic attack by human polymorphonuclear leucocytes (PMNLs).

In this report, we examined inter-related factors that affect the susceptibility of different culture morphotypes of *L. monocytogenes* to phagocytosis and survival in human PMNLs.

Materials and methods

Bacterial strains and growth media

The *Listeria* strains used in the study were, if not otherwise indicated, derived or obtained from the National Collection of Type Cultures (NCTC), Public Health Laboratory Service (PHLS), Central Public Health Laboratory, Colindale, UK (Table 1). Autoagglutinable MCR2 and FR2 isolates of *L. monocytogenes* exhibiting rough culture phenotypes were obtained from Dr Jim McLauchlin, Food Safety Microbiology Laboratory, PHLS, Colindale, UK. The FR2 clinical strain of *L. monocytogenes* was obtained from a 72-year-old male with sepsis and pyrexia. The rough filamentous variant FR1 was derived from a wild-type smooth form of *L. monocytogenes* NCTC 4885 after exposure to adverse culture conditions created by high-intensity pulsed-plasma gas discharge treatments according to the method described in Rowan *et al.* (2008).

The *Listeria* cells were grown for 48 h at 37 °C on brain heart infusion agar (BHIA), 7% (v/v) sheep blood agar (BA) and on tryptone soya agar supplemented with 15 μg mL^{-1} hemin (TSHA) and tryptone soya agar without hemin (TSA), before challenging with human PMNLs. Hemin supplements were purchased from Sigma-Aldrich Chemical Co. Ltd, dissolved in hot water, adjusted to pH 7 and filter sterilized before adding to TSA as per the recommendations of Tsai & Hodgson (2003). Stored bacteria were maintained at 4 °C on TSA slopes until use; fresh isolates of each test strain were also recultured every 2 weeks from original vials that were kept at −70 °C in the Microbank™ system.

Biochemical and physiological methods

Catalase production was determined by applying a drop of 3% H_2O_2 to the colonies and observing the occurrence of O_2 bubbles, as described elsewhere (Bubert *et al.*, 1997). The Christie, Atkins, Munch–Petersen (CAMP) test was performed using standard procedures by streaking out

Table 1. *Listeria monocytogenes* strains used and their characteristic morphological and physiological properties identified in this study

				Cell morphology and physiological properties					
Strain	Serotype	Reference strain no.	Origin	Tumbling motility	Cell form	Cell-type designation	Cell length (μm)*	Colony area (μm^2)†	Titer by ELISA ($A_{492\ nm}$) with anti-CwhA mAb‡
S1	4b	NCTC 11994	Adult meningitis	+	Single, paired cells	Wild-type smooth	3.0 ± 1.0 A	(1.9 ± 0.6) × 10^5 A	0.61 ± 0.05 B
S2	4b	NCTC 9863	Infantile meningitis	+	Single, paired cells	Wild-type smooth	3.7 ± 1.2 A	(2.3 ± 0.8) × 10^5 A	0.55 ± 0.08 B
MCR1§	1/2a	SLCC 5764	Kathariou *et al.* (1987)	+	Long cell chains	MCR variant	42.3 ± 20.9 B	(3.9 ± 0.7) × 10^6 B	0.23 ± 0.06 A
MCR2	¶	L7071	Dried custard powder	—	Long cell chains	MCR variant	56.1 ± 32.3 B	(1.7 ± 0.8) × 10^6 B	0.25 ± 0.03 A
FR1	4b		Derived from NCTC 4885‖	—	Long filaments	FR variant	65.2 ± 37.9 B	(9.3 ± 0.6) × 10^6 C	0.73 ± 0.06 C
FR2	¶	L1342	Blood culture	—	Long filaments	FR variant	63.5 ± 38.3 B	(9.5 ± 1.1) × 10^6 C	0.59 ± 0.05 B

*Mean length (μm) from 10 measurements (±SD). Single and paired cells were measured and averaged for the smooth-cell forms. Values in the same column followed by the same letter did not differ at the $P < 0.05$ level, while values with different letters differed at the $P < 0.05$ level.

†Mean of area measurements for 10 colonies grown for 48 h on LSA plates.

‡$OD_{492\ nm}$ values > 0.1 were considered a positive result. BHI broth controls gave a value of 0.004 ± 0.01.

§Derived from *Listeria monocytogenes* Mackaness (SLCC 5764).

¶Autoagglutinable cultures and therefore not serotypable.

‖Derived from *L. monocytogenes* NCTC 4885 after a 5-min exposure to high-intensity, pulsed-plasma, gas discharge decontamination conditions.

+, a positive motility test for *L. monocytogenes*.

bacteria perpendicular to *Staphylococcus aureus* on 7% sheep BA plates containing and observing zones of augmented hemolysis, as described elsewhere (Rowan *et al.*, 2000). A characteristic blue-green sheen from colonies by obliquely transmitted light and tumbling motility of *Listeria* cells was observed as described elsewhere (Rowan *et al.*, 2000). The commercial biochemical API *Listeria* test (BioMerieux, Marcy l'Etoile, France) was used according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA) for the detection of CwhA protein

Detection of CwhA protein (formerly p60) in all test *Listeria* strains using indirect ELISA involved the addition of 100 μL of cell-free supernatant per well of microtiter plates (supernatant from an overnight culture and harvested by centrifugation) and incubation for 2 h at 37 °C. Coated proteins were washed three times with wash buffer [phosphate-buffered saline (PBS) containing 0.1% v/v Tween 20] and the *L. monocytogenes*-specific monoclonal antibody CwhA-mAb K3A7 was added. This mAb was generated against the *L. monocytogenes*-specific epitope, peptide D, of the p60 protein, which has been described previously (Bubert *et al.*, 1997). Generation of K3A7 was achieved using standard protocols (Kuhn & Goebel, 1989). The anti CwhA-mAb was prepared as a tissue culture supernatant diluted 1/200 (v/v) in wash buffer and incubated for 1 h at room temperature. The microtiter wells were washed three times with wash buffer, and sheep anti-mouse horseradish peroxidase conjugate (Sigma-Aldrich Chemical Co. Ltd) was added at 100 μL per well at a dilution of 1/1000 in wash buffer and incubated for 1 h at room temperature. Excess conjugate was washed five times with wash buffer and the substrate SIGMA FAST™ OPD tablets (Sigma-Aldrich Chemical Co. Ltd) was added at 100 μL per well with 0.5 h of incubation at room temperature. The $A_{492\ nm}$ was measured after the addition of 50 μL per well 3 M H_2SO_4.

Cell or filament length and colony appearance determination

Overnight cultures of all the *L. monocytogenes* strains described in Table 1 were separately incubated in BHI broth at 37 °C with aeration. At various time intervals, the lengths of the cells were determined using image analysis (Nikon Optiphot-2 microscope that was connected to a Solitaire 512 Image Analyzer, Seescan Plc.). Ten cells were measured per sample. Overnight cultures were also grown at 37 °C on *Listeria*-selective agar (LSA; Oxford formulation; Oxoid) to investigate differences in colony appearance. The area (μm^2) of 10 colonies per sample was measured using the image analysis system mentioned above.

Electron microscopy

Cells were grown to the stationary phase (30 h) on BA plates, washed twice with PBS and resuspended in sterile-distilled water before application to formvar-coated grids. After the grid was dried, one drop of a solution containing 3% v/v tungstophosphoric acid and 0.3% v/v sucrose (pH 6.8–7.4) was added. The solution was removed after 30–60 s, and the grid was dried and examined on a Zeiss 902 transmission electron microscope.

Measurement of bacterial susceptibility to opsonophagocytosis

Preparation of bacteria

Bacteria were separately grown for 16 and 30 h on BA, BHIA and TSYEA at 37 °C. Bacteria were then harvested, washed and standardized spectrophotometrically ($OD_{560\,nm}$) so that suspensions containing 1×10^8 CFU mL^{-1} (PMNL ingestion studies) or 5×10^8 CFU mL^{-1} (measurement of respiratory burst) were available for investigation.

Bacterial opsonization

The standard bacterial suspensions were opsonized in 1 mL of 10% (v/v) normal human pooled serum containing 0.1% gel-HBSS (Hanks basal salt solution with 0.1% gelatin) for 15 min at 37 °C; the opsonized cells were harvested by centrifugation and resuspended in diluent (PBS, pH 7.1) at the original concentration. Unopsonized bacteria (no serum) were used for comparison.

Isolation of neutrophils (PMNLs)

PMNLs were isolated from 20 to 30 mL of fresh blood taken from healthy human donors in heparin vacutainers (and inverted to mix) using density gradient centrifugation through Ficoll-Hypaque (Polymorphprep; Nycomed, Amersham, UK) as per Gemmell & Ford (2002). The neutrophil-rich layer was harvested and washed gently with 0.1% gel-HBSS before standardization to a concentration of *c.* 1×10^7 cells mL^{-1} (as determined using a hematocrit chamber). Purity was $\geq$95% and viability, as measured by exclusion of trypan blue (0.1% solution), was also $\geq$95%.

Phagocytosis and bacterial killing by PMNLs

The ability of PMNLs to phagocytose and kill opsonized cells of *L. monocytogenes* was assessed using the previously published method of Gemmell & Ford (2002). Briefly, 0.1 mL of a suspension of opsonized or nonopsonized bacteria grown separately on BA plates for 16 and 30 h were added to duplicate polypropylene vials, followed by the addition of 0.1 mL of PMNLs. The vials were then incubated for various time intervals up to and including 45 min at 37 °C in an orbital shaking incubator at 250 r.p.m. Experiments were performed using a bacteria/PMNL ratio of 50 : 1. Immediately after removal from the incubator at the indicated times, 2.5 mL of ice-cold 0.1% gel-HBSS was added and the phagocytic cells were washed three times in PBS by differential centrifugation at 1000 ***g*** (4 °C) for 5 min. Washing and shaking with PBS was carried out to remove trapped or loosely adherent bacteria from neutrophils. Slide preparations were then made of a selection of the bacteria/PMNL mixtures, as follows: after the washing stages, the reaction mixture was resuspended in 50 μL of PBS and centrifuged at 21 ***g*** for 5 min on a silane-coated glass slide using a cytocentrifuge (Cytospin 2, Shandon, Southern Products Ltd, Runcorn, UK) in order to visually assess phagocytic cells. After drying for 10 min, the slides were stained with Giemsa stain and examined microscopically at $\times$1000 magnification. The phagocytic index was determined as percent positive macrophages (those containing more than one bacterium) times the mean number of bacteria per positive macrophage as described previously by Alford *et al.* (1991).

The bacterial killing process was measured by constituting mixtures as for the phagocytosis assay above and determining CFUs at specific times. Four sets of vials were made up for each assay, one to be sampled immediately upon the addition of PMNL (zero time) and the other three to be sampled at 15, 30 and 45 min, respectively. Volumes of 0.1 mL of opsonized or nonopsonized bacteria were added to each vial, followed by the addition of 0.1 mL of PMNL. Then, after the desired incubation intervals (0, 15, 30 or 45 min), 0.1 mL of cold sterile water was added to each vial; the vials were mixed thoroughly, and appropriate dilutions were prepared. Samples of these dilutions were pipetted into sterile Petri dishes, and 15 mL of sterile molten BHIA was added, mixed well and allowed to solidify. Survivors (expressed in terms of CFU) were enumerated after an overnight incubation at 37 °C and percent change in CFU at 15, 30 and 45 min, relative to CFU at zero time, was calculated. Experiments were repeated in triplicate on at least 3 separate days with PMNL from different normal donors.

The phagocytic process was measured using the peak chemiluminescence assay of Gemmell & Ford (2002), which follows the respiratory burst in neutrophils exposed to serum-opsonized (and nonopsonized) bacteria that had been grown separately on BA, BHIA or TSHA and TSA plates. Opsonized bacteria (5×10^7 CFU) were added to a reaction mixture consisting of 5×10^5 PMNL and 50 μL of 1×10^{-5} M luminol in the dark. Light release was then measured from the chemiluminescence counter (BioOrbit 1253, BioOrbit, Turku, Finland) over a 45-min period and peak response (mV) was noted.

Statistical methods

All experiments in this study were performed in triplicate, and results are reported as averages with SDs. The influence of strain type, culture form (wild-type smooth vs. cell-chain and filamentous rough forms), opsonization and plating medium on the uptake and survival of *L. monocytogenes* cells in PMNLs was determined at the 95% or greater confidence intervals using ANOVA (one-way or balanced model) with MINITAB software Release 13 (Minitab Inc., State College, PA).

Results

Confirmation of the morphological and physiological characteristics of different culture types isolated previously from clinical and food samples

All the bacterial strains described in Table 1 were identified as *L. monocytogenes* by establishing the characteristic morphological, physiological and biochemical properties associated with this pathogen. All cultures produced catalase, were CAMP test positive with *S. aureus* and were identified as *L. monocytogenes* using API *Listeria* biochemical profiling. Confirmation of species identification was performed by analysis of culture supernatant for CwhA protein by indirect ELISA with an *L. monocytogenes*-specific anti-CwhA mAb (Table 1).

The cell and colony appearances of all rough variants were shown to differ significantly from those of the wild-type *L. monocytogenes* strains, which had a typical smooth morphology. Unlike wild-type smooth strains, whose cells have a characteristic coccobacillus appearance (*c.* 0.5 μm in diameter × 2 μm in length), cell types associated with the rough variants were shown to be atypically long. Some rough variants consisted of unseptated or paired filaments that measured up to 103 μm in length (designated FR variants), whereas others formed long chains that were up to 88 μm in length and consisted of multiple cells of similar size (designated MCR variants) (Table 1). Rough variants isolated from clinical specimens or derived under conditions of stress produced by exposure to pulsed-plasma gas discharge showed the FR filamentous phenotype. The mean cell lengths for the various culture variants of *L. monocytogenes* were shown to be 3.3 ± 1.1 (wild-type normal S type), 49.2 ± 26.6 (MCR variant) and 64.3 ± 38.1 (FR variant). MCR and FR variants were found to be incapable of the characteristic tumbling motility and formed irregular or rough colonies that no longer produced a blue-green sheen upon oblique illumination. Image analysis data showed that irregular rough colonies consistently had areas (μm^2) and appearances ($P < 0.05$) different from that of smaller, wild-type form colonies after 48 h of growth on LSA plates (Table 1). Spontaneously occurring MCR1 variant and food sample isolate MCR2 with a rough colony appearance exhibited similar phenotypes, tending to form long chains in which septum formation between individual cells still occurred, but the cells were not separate.

Indirect ELISA studies (Table 1) with an anti-CwhA mAb showed that these MCR variants secrete a considerably reduced amount of a peptidoglycan hydrolase protein. The addition of partially purified CwhA led to a decay of the cell chains (mean 49.2 ± 26.6 μm) to normal size (mean 3.3 ± 1.0 μm) within 3 h of treatment. The blood culture isolate FR2 and the FR1 variant of *L. monocytogenes* derived from the wild-type S form culture after exposure to pulsed-plasma gas discharge treatment also exhibited a rough filamentous cell appearance. However, unlike MCR variants that secreted diminished levels of cell-free CwhA, indirect ELISA studies showed that FR variants produced wild type or greater amounts of Cwha, indicating that the latter FR morphotypes were type II in nature (Table 1). The addition of partially purified CwhA from wild-type *L. monocytogenes* to FR variants did not decay the lengths of the filaments (mean 64.3 ± 38.1 μm) to the normal *Listeria* cell size.

Effect of opsonization and composition of bacterial growth medium on the susceptibility of different culture forms of *L. monocytogenes* to phagocytosis by human PMNLs

The induction of a respiratory burst by human PMNL following exposure to different culture forms of *L. monocytogenes* was measured as the maximal chemiluminescence (mV) response, usually occurring 15–20 min after addition of the bacterium/luminol reaction mixture in the dark. The level of respiratory burst of PMNL exposed to 10% opsonized bacteria was significantly different ($P = 0.00003$) from similar nonopsonized bacteria (Table 2). Studies revealed that prior growth of all test bacteria on media containing or supplemented with iron augmented respiratory burst activity in PMNLs ($P = 0.0016$), with mean (mV) measurements of 56.2 ± 4.4, 45.6 ± 5.6, 43.0 ± 4.7 and 34.3 ± 3.3 for bacteria grown on BA, BHIA, TSHA and TSA, respectively (Table 2). Albeit in lower measured amounts, a similar pattern of respiratory burst activity was observed for nonopsonized bacteria with mean (mV) measurements of 14.4 ± 3.6, 7.8 ± 2.7, 7.6 ± 1.7 and 5.5 ± 1.3 for BA, BHIA, TSHA and TSA, respectively. Wild-type smooth *Listeria* elicited the greatest respiratory burst in PMNLs ($P = 0.008$) compared with MCR or FR variants. No significant difference was observed between rough MCR and FR culture variants in eliciting maximal chemiluminescence responses ($P = 0.497$). However, wild-type smooth S2 elicited a stronger respiratory burst response compared with S1 ($P = 0.006$), while there was no difference in the chemiluminescence responses between individual strains of either

Table 2. Effect of *Listeria monocytogenes* strain type, culture morphotype, opsonization and composition of agar media on the maximal chemiluminescence response (mV) elicited by phagocytosing PMNLs

	Maximal chemiluminescence (mV)† after ingestion of different morphotypes of *L. monocytogenes* by PMNLs							
	Opsonized cells (10% serum)				Nonopsonized cells (0% serum)			
Strain*	BA	BHIA	TSHA	TSA	BA	BHIA	TSHA	TSA
S1	62.7 ± 4.1 C	54.3 ± 8.3 D	48.1 ± 4.4 D	39.6 ± 3.4 E	12.9 ± 2.3 G	7.6 ± 1.4 H	7.2 ± 1.4 H	4.6 ± 1.2 I
S2	80.8 ± 7.1 A	70.8 ± 3.5 B	64.6 ± 5.3 C	52.3 ± 4.5 D	23.5 ± 8.8 F	9.9 ± 3.8 G	9.1 ± 1.8 G	6.9 ± 1.4 H
MCR1	45.2 ± 3.4 D	37.5 ± 5.0 E	35.1 ± 4.1 F	30.1 ± 2.7 F	13.1 ± 4.7 G	5.8 ± 2.0 H	7.5 ± 2.1 H	4.1 ± 1.1 I
MCR2	47.8 ± 3.9 D	37.0 ± 6.4 E	39.8 ± 5.4 E	30.0 ± 2.9 F	10.8 ± 1.1 G	7.3 ± 6.8 H	6.7 ± 2.0 H	4.1 ± 1.2 I
FR1	50.3 ± 2.9 D	39.3 ± 4.8 E	33.3 ± 4.1 F	26.2 ± 4.1 F	11.4 ± 2.7 G	6.8 ± 1.3 H	6.5 ± 1.3 H	6.2 ± 1.8 H
FR2	50.6 ± 5.5 D	34.9 ± 5.8 E	37.5 ± 5.3 E	28.1 ± 2.2 F	14.9 ± 2.5 G	9.3 ± 1.4 G	8.5 ± 1.4 H	6.7 ± 1.3 H

*Description of the strains provided in Table 1.
†Mean (± SD) of triplicate samples measured from triplicate trials. Values (mV) having different uppercase letters differ at the $P < 0.05$ level.
BA and BHIA contain a source of iron, whereas TSHA is artificially supplemented with hemin.

MCR or FR variants ($P = 0.513$). Visualization of the bacterium/phagocyte mixture using light microscopy revealed greater numbers of intracellular smooth-type bacteria compared with PMNLs containing MCR or FR variants. The mean numbers of bacteria ingested by phagocytosing macrophages after 45-min exposure were shown to be 12 ± 4, 7 ± 4 and 6 ± 3 for the smooth, MCR and FR cell types, respectively (Fig. 1). A quantitative measurement of PMNL-ingested bacteria (phagocytic index) revealed significant differences ($P < 0.05$) between the wild-type smooth form (984 ± 286) compared with similarly exposed rough MCR (441 ± 213) and FR (330 ± 151) variants (Fig. 1).

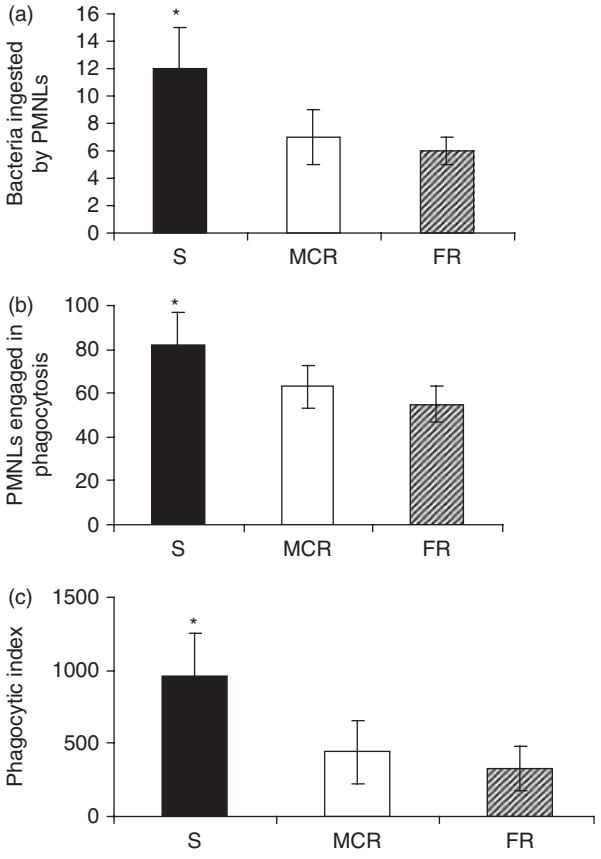

Fig. 1. Influence of bacterial load on the phagocytosis of different morphotypes of *Listeria monocytogenes*: (a) number of bacteria ingested by phagocytosing PMNLs, (b) proportion of PMNLs engaged in phagocytosis, and (c) phagocytic index (mean ± SD of three experiments). *Statistical difference at the $P < 0.05$ level.

Survival of different opsonized culture variants of *L. monocytogenes* in human PMNLs

Survival of different opsonized (10% serum) culture forms of *L. monocytogenes* in PMNLs was examined over a 45-min exposure period (Fig. 2). A clear pattern emerged where the percentage of bacterial survivors post-PMNL ingestion decreased over the 45-min exposure for all *Listeria* culture forms studied. While wild-type smooth and FR variants exhibited a similar percentage survival in PMNLs, strains belonging to MCR variant were significantly less resistant to exposure to deleterious stresses associated with internalization in PMNLs. Greater survival of all culture forms of *L. monocytogenes* occurred in PMNLs where bacteria were either grown before on BA plates (Fig. 3) and/or when older 30-h cultures were used compared with younger 16-h strains (Fig. 4) ($P < 0.05$). Interestingly, less pronounced survival in PMNLs occurred after 45-min exposure, where bacteria had been grown before on TSA supplemented with hemin (Fig. 5) compared with similar samples grown on BA (Fig. 3b) plates. However, although the mean survival percentages were generally higher for the majority of morphotypes studied compared with prior growth of similar samples on TSA alone (Figs 3 and 5), only S2 grown on BA was observed to be significantly different at the $P < 0.05$ level after 45 min of exposure to PMNLs (Fig. 3b). Greater survival ($P < 0.05$) occurred for all S and FR morphotypes studied after shorter

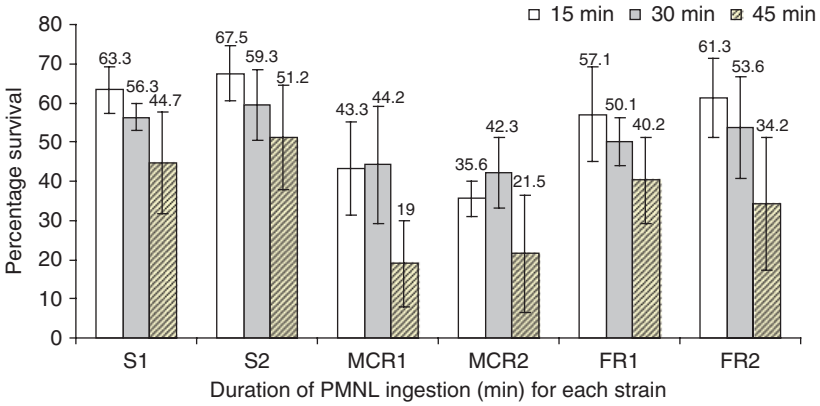

Fig. 2. Percentage survival of different morphotypes of *Listeria monocytogenes* in human PMNLs challenged over a 45-min exposure period (mean ± SD of three experiments).

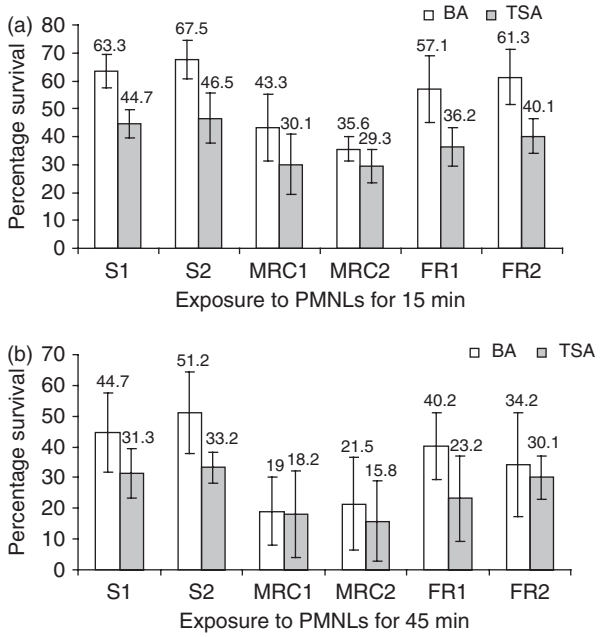

Fig. 3. Effect of growth of bacteria on BA media containing a source of iron on the survival response (percentage) of different morphotypes of *Listeria monocytogenes* in human PMNLs challenged for (a) 15 min and (b) 45 min (mean ± SD of three experiments).

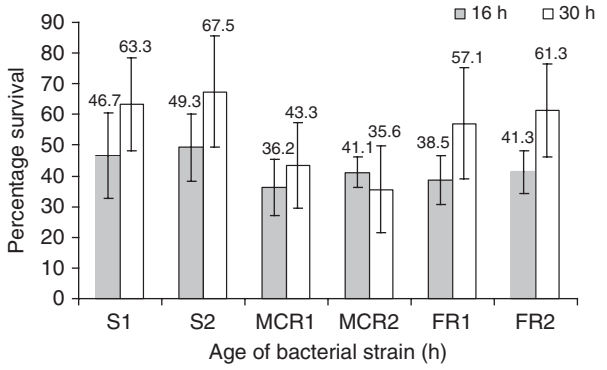

Fig. 4. Effect of bacterial cell age on the survival response (percentage) of different morphotypes of *Listeria monocytogenes* in human PMNLs challenged over a 45-min exposure period (mean ± SD of three experiments).

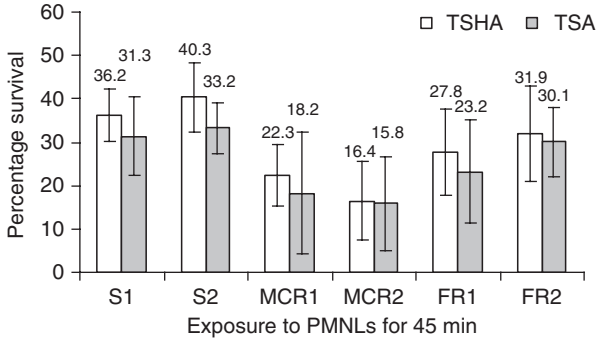

Fig. 5. Effect of supplementation of TSA with hemin on the survival response (percentage) of different morphotypes of *Listeria monocytogenes* in human PMNLs challenged for 45 min (mean ± SD of three experiments).

durations of exposure to PMNLs, where the latter had been grown before on BA plates (Fig. 3a).

Discussion

This study demonstrated that human PMNLs are capable of engulfing and internalizing different cell or filament morphotypes of *L. monocytogenes* that were isolated from clinical specimens and food samples. However, the efficacy of this phagocytosis process was shown to depend on many factors namely bacterial strain, culture form (normal smooth vs. atypical rough variants), degree of opsonization and the composition of bacterial culture media used before uptake by PMNLs. The much smaller normal cell type of *L. monocytogenes* (*c.* 2 μm in length) was shown to be much more susceptible to phagocytic uptake compared with

atypical rough chains of cells or filaments that measured up to *c.* 100 μm in length. Studies reporting previously on the pathogenicity of different culture variants of *L. monocytogenes* are very limited. Wilder & Edberg (1973) appear to be the first researchers to report on the interaction of regular-sized smooth and atypical rough cells of *L. monocytogenes* with normal mouse peritoneal macrophages in culture and revealed that after 24 h the latter rough variants were almost completely killed, whereas the smooth strain tended to show complete survival. The authors did not indicate whether the rough variants constituted a filamentous or the long cell chain of cell arrangement. However, the findings of Wilder & Edberg (1973) appear to markedly contrast similar PMNL studies carried out earlier by Mackaness (1962), where the latter reported that macrophages initially infected with *L. monocytogenes* released their content of pathogens at 20 h. In the present study, the viability of all culture morphotypes of *L. monocytogenes* was reduced within 45 min of engulfment by human PMNLs.

Visual observation of *L. monocytogenes* S2 cells engulfed in PMNLs revealed that some bacteria had converted to longer chains of cells. The numbers of internalized *Listeria* were shown to be < 16 cells or filaments in positive PMNLs. Kingdon & Sword (1970) previously reported that *L. monocytogenes* killed the host macrophage when the intracellular population increased to approximately 16 bacteria per cell. Hardy *et al.* (2004) also observed the conversion to chains of cells from normal-sized *L. monocytogenes* cells during replication in murine gall bladder. Interestingly, chain morphology was not observed during intracellular growth of wild-type bacteria in an ordinary broth culture. The significance of bacterial growth in the gall bladder with respect to the pathogenesis and the spread of listeriosis depends on the ability of the bacterium to leave this organ and be disseminated to other tissues and into the environment. Rowan *et al.* (2000) previously demonstrated that atypical rough filaments of *L. monocytogenes*, isolated from clinical specimens and food samples, demonstrated wild-type levels of adherence, invasion and cytotoxicity to human epithelial HEp-2, Caco-2 and HeLa cells.

Use of 10% serum in the present study augmented phagocytosis of all different culture variants of *L. monocytogenes*, which appears to agree with findings from recent studies of Vahidy & Jehan (2006), who demonstrated the role of immune serum in augmenting the *in vitro* engulfment of typical short cells of *L. monocytogenes* by PMNLs of rabbits. Growth of different variants of *L. monocytogenes* on BA resulted in increased respiratory burst activity and bacterial survival in human PMNLs. This also correlated with visible inspection of bacteria/phagocytes mixtures (phagocytic indices) that revealed greater numbers of internalized *Listeria* cells in PMNLs when these bacteria were grown on BA compared with similar cells grown on TSA plates before PMNL challenge. While BA contains a source of iron, supplementation of TSA with hemin did not produce similar levels of respiratory burst activity in engulfed bacteria, indicating that other constituents in BA (and to a lesser degree in BHIA) contribute to the augmented chemiluminescence responses. The presence of hemin augmented respiratory activity in PMNLs compared with the use of TSA alone for many opsonized morphotypes alone, indicating that prior microbial exposure to iron in bacterial growth media influenced respiratory burst activity in PMNLs. However, supplementation of bacterial growth media with hemin did not significantly enhance survival in this present study. The augmented respiratory burst activity in the presence of iron may be indirectly mediated by the Haber–Weiss–Fenton and related reactions (Yuan *et al.*, 1995). In these reactions, hydroxyl radicals are generated from hydrogen peroxide in the presence of Fe^{2+}, which is oxidized to Fe^{3+}. Generation of more hydroxyl radicals and Fe^{3+} results from the reaction between superoxide and Fe^{2+}. It is likely that, in activated macrophages, the concentration of superoxide ions and hydrogen peroxide increases due to the above-summarized reactions. It is also well documented that the level of iron determines the expression of several virulence factors and that survival of bacteria during infection also depends on the ability to interact with and acquire iron from the host (Braun, 2005). It has been reported previously that during listerial infection, the availability of iron determines the survival and invasiveness of the bacterium (Rea *et al.*, 2004).

It is interesting to note that the filamentous morphotype of *L. monocytogenes* can also arise after exposure to deleterious conditions associated with novel food processing or environmental decontamination (Giotis *et al.*, 2007). In the present study, we describe the occurrence of an FR1 variant that was generated after sublethal exposure to high-intensity pulsed-plasma gas discharge conditions, which is a technique similar in design to that of high-intensity pulsed electric fields with the inclusion of a gas-sparging stage (Rowan et al., 2008). Hahn *et al.* (1999) suggest that the switch to the filamentous state in bacteria may be a survival response defending the latter against flagellate grazing. While human PMNLs were capable of engulfing type II filaments of *L. monocytogenes*, it is not apparent from this present study as to whether or not there was an upper limit or threshold in terms of filament size. Although lower numbers of rough cell chain or filament types were engulfed by macrophages compared with normal short cell types of *L. monocytogenes*, studies were not carried out to elucidate the ability of macrophages to engulf *Listeria* based on the degree of filament or chain length. Different culture variants of microbial pathogens, including *L. monocytogenes*, may arise from exposure to harsh growth conditions that may alter their morphological, physiological and virulence

characteristics. Prior exposure to hostile growth conditions can protect these bacteria from subsequent exposure to lethal levels of the same or different stresses (Aldsworth *et al*., 1999; Rowan, 1999; Hardy *et al*., 2006). During the parasitic lifecycle, *L. monocytogenes* must cope with a series of challenges that arise during different stages of infection. These challenges include low nutrient availability, resistance to various physical stresses (e.g. temperature, acid, bile; Gahan & Hill, 2005) and the host immune system (Pamer, 2004). Stress resistance and dramatic switches in cellular morphology and survival characteristics in *L. monocytogenes* are undoubtedly governed by the expression of specific gene products, which is possibly coordinated and regulated by PrfA and by other presently unknown transcriptional controlling factors (Hardy *et al*., 2006; Riedel *et al*., 2009). Interestingly, the latter authors also observed that differences in culture media formulations used for biofilm production (i.e. use of 10-diluted BHI vs. TSB) had a dramatic effect on the ability of an Δ*agrD*-mutant strain of *L. monocytogenes* to form biofilms. A clear trend also emerged where older cultures of *L. monocytogenes* survived better in human PMNLs compared with similarly prepared and exposed younger variants. Previous researchers have also demonstrated that pathogens in their stationary growth phase survive adverse conditions associated with food processing, in part, due to the fact that their metabolic processes have slowed down and are less susceptible to the lethal action of extrinsic stresses (Rowan, 1999; Giotis *et al*., 2007).

In conclusion, while human PMNLs are capable of engulfing different culture morphotypes of *L. monocytogenes*, typical short and atypical type II filamentous types of this pathogen appear to survive this immune challenge better than multiple cell chain variants that secrete reduced levels of the peptidoglycan hydrolase CwhA protein. All esculin-positive culture isolates growing on LSA plates should be tested further to confirm their identity using an immunological or equivalent molecular-based probe that is species specific for *L. monocytogenes*.

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