Monoclonal antibodies binding to the cell surface of *Listeria monocytogenes* serotype 4b

Min Lin, Dobrila Todoric, Maria Mallory, B. Steven Luo, Erin Trottier and Hanhong Dan

Animal Diseases Research Institute, Ottawa, Ontario, Canada K2H 8P9

Correspondence

Min Lin

linm@inspection.gc.ca

Serotype 4b strains of the food-borne pathogen *Listeria monocytogenes* are responsible for a large portion of sporadic listeric infections and all major food-borne listeriosis outbreaks in humans. Hybridomas were produced from three fusions with lymphocytes of ND4 mice immunized either with the insoluble antigens of *L. monocytogenes* serotype 4b or with formalin-killed bacterial cells and screened for monoclonal antibodies (mAbs) reactive to *L. monocytogenes* serotype 4b. A set of 35 mAbs was identified by ELISA as having reactivity with both the insoluble antigen fraction and the whole-cell antigens. Thirteen of these mAbs belonged to immunoglobulin subclass G1 (IgG1), fifteen were IgG2a and seven mAbs were IgM. Only 20 out of the 35 mAbs were capable of detecting protein bands of various sizes ranging from 20 to 88 kDa in Western blots. Two of these mAbs, M2365 and M2367, were capable of binding to cell-surface antigens of live *L. monocytogenes* serotype 4b, as demonstrated by immunofluorescence microscopy and immunogold transmission electron microscopy. Immunofluorescence microscopy showed that M2365 and M2367 failed to bind to the cell surfaces of *Escherichia coli* O157:H7, *Salmonella enterica* (serotype Typhimurium DT104) or *Campylobacter jejuni*. Evaluation of the cross-reactions of all 35 mAbs with whole-cell antigens of *E. coli* O157:H7, *S. Typhimurium*, *C. jejuni* and *Listeria innocua* by ELISA indicated that the majority of the mAbs, including M2365 and M2367, did not cross-react with *E. coli* O157:H7, *S. Typhimurium* or *C. jejuni* and showed no or a very weak reaction with *L. innocua*. Furthermore, M2365 and M2367 showed no reaction with whole-cell antigens derived from *L. monocytogenes* serotypes 1/2a, 1/2b and 3a, and from *Listeria grayi*, *Listeria ivanovii* and *Listeria seeligeri*, in an ELISA. Collectively, these data suggest that M2365 and M2367 have potential use in the development of immunological methods of laboratory diagnosis for *L. monocytogenes* serotype 4b in clinical or food samples.

Received 23 August 2005

Accepted 8 November 2005

INTRODUCTION

Listeria monocytogenes is a Gram-positive, rod-shaped, facultatively anaerobic, intracellular bacterium that causes infection (listeriosis) in man and animals. Individuals most susceptible to the pathogen include neonates, pregnant women, the elderly, immunocompromised persons and those with impaired cell-mediated immunity such as human immunodeficiency virus-infected patients. Common clinical syndromes of listeriosis include septicaemia, meningitis, encephalitis, febrile gastroenteritis, abortion and stillbirths. The organism is ubiquitous in nature and is found in water, soil and vegetation. It grows and survives in extreme conditions, such as a wide range of temperatures from -1.5 to 50°C , a pH range of 4.3–9.6 (Donnelly, 2001) and high salt concentrations, and can tolerate freezing and drying, making it an ideal candidate for transmission through food and rendering it a difficult organism to eliminate from food

chains. Transmission from mother to fetus is the only documented form of person-to-person transmission (Lorber, 1997). Food-borne listeriosis has become a great concern to public health and food industries due to a number of outbreaks and sporadic cases caused by foods contaminated with *L. monocytogenes* (Lorber, 1997; Schlech, 2000; Donnelly, 2001). Although listeriosis is a rare human illness, it is a leading cause of mortality from a food-borne pathogen (Mead *et al.*, 1999). This fact emphasizes the need to develop a rapid detection system for *L. monocytogenes*.

In comparison with culture methods followed by biochemical tests, nucleic acid-based and antibody-based methods have been demonstrated to be very promising for rapid detection of *L. monocytogenes* in food and clinical samples (Ralovich, 1993; Norton, 2002). Monoclonal antibodies (mAbs) with high specificity and affinity for *L. monocytogenes* are essential for the development of an immunoassay to detect this pathogen. The production of mAbs to *Listeria* spp. has been described in a number of studies (Ziegler & Orlin, 1984; Farber & Speirs, 1987; McLauchlin *et al.*, 1988;

Abbreviations: H+L, heavy and light; IC, insoluble cellular; WC, whole cell.

Bhunja *et al.*, 1991; Bhunia & Johnson, 1992; Torensma *et al.*, 1993; Kathariou *et al.*, 1994; Loiseau *et al.*, 1995). However, none of the mAbs reported to date are specific for *L. monocytogenes* strains with the exception of mAb EM-7G1 described by Bhunia & Johnson (1992). Almost all human infections are attributed to serotypes 4b, 1/2a and 1/2b (Lorber, 1997; Donnelly, 2001), with serotype 4b strains accounting for approximately 40 % of cases (Rocourt & Bille, 1997). Thus, immunoassays specific for serotype 4b strains are very useful for epidemiological investigations. Kathariou *et al.* (1994) reported two mAbs, c74.33 and c74.180, that showed a high degree of specificity for serotype 4b strains, but also recognized serotypes 4d and 4e. It would be of great interest and is also of necessity to develop mAbs for the purpose of developing a laboratory diagnostic test specific for serotype 4b strains.

We are interested in immunogenic surface proteins of *L. monocytogenes*, which are less characterized and understood. Study of such proteins may provide new insights into the mechanisms of *Listeria* pathogenesis, virulence and immunity. As such, our laboratory has initiated a series of studies aimed at the identification and characterization of the immunogenic surface proteins of *L. monocytogenes* (Yu, 2004), including the production and characterization of mAbs to *L. monocytogenes* as described in this paper. The reactivity of these mAbs with several important food-borne pathogens (*Escherichia coli* O157:H7, *Salmonella enterica*, *Campylobacter jejuni*), several *Listeria* species and *L. monocytogenes* serotypes was examined using ELISA, Western blots, immunofluorescence microscopy and immunogold electron microscopy to identify mAbs binding specifically to the cell surface of *L. monocytogenes* serotype 4b. These mAbs may be useful for probing the surface antigenic composition of *L. monocytogenes* and may also have potential use in the detection of *L. monocytogenes* for epidemiological investigations, food safety surveillance, diagnosis, serotyping and pathological studies.

METHODS

Chemicals and reagents. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L chain) and 12 nm colloidal gold-conjugated goat anti-mouse IgG (H+L chain) were purchased from Jackson ImmunoResearch Laboratories. Fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (H+L chain) was bought from Zymed Laboratories. Protein standards (pre-stained), nitrocellulose membranes, the protein assay kit and the HRP-conjugate substrate kit were obtained from Bio-Rad. DNase I and RNase A were purchased from Roche Diagnostics; a protease inhibitor cocktail, 2,2'-azino-bis(-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and Freund's adjuvant were from Sigma-Aldrich. The mAb M2165 (tissue-culture fluid) directed against the E^{rms} protein of *Classical swine fever virus* has been described previously (Lin *et al.*, 2005) and was used as a negative control for ELISA, Western blots, immunofluorescent labelling and immunogold labelling studies. All other chemicals were of commercially available analytical grade.

Bacterial culture. *L. monocytogenes* LI0521 (serotype 4b) and *Listeria innocua* were maintained on trypticase soy blood agar plates at 4 °C. A single *Listeria* colony was inoculated into Luria-Bertani

(LB) broth containing 50 mM MOPS (pH 7.5) and cultured at 37 °C for 16–18 h. The *Listeria* cell number was determined from the absorbance reading, with an OD₆₂₀ value of 0.61 being equivalent to 1×10^9 bacteria ml⁻¹ (Schlech, 1993), and confirmed by plating serial dilutions of cells onto LB agar plates. *E. coli* O157:H7 ATCC 43889 and *S. enterica* SA03-1907 serovar Typhimurium DT104 (*S. Typhimurium*), maintained on LB agar plates at 4 °C, were used to inoculate LB broth and cultured at 37 °C for 16–18 h. The cell density of *E. coli* was calculated based on an OD₆₀₀ value of 1 being equivalent to 1×10^9 cells ml⁻¹ (Ausubel *et al.*, 2005). The number of *S. Typhimurium* was estimated by plating serial dilutions of cells onto LB agar plates. A pure culture of *C. jejuni* NCTC 11168, from the bacteriology stock culture collection at the Animal Diseases Research Institute was streaked onto 25 cysteine heart blood agar plates to produce well-isolated colonies. The plates were placed into anaerobic jars and incubated microaerophilically at 35 °C for 3 days. The microaerophilic conditions were generated by evacuating and filling the air content in the jars three times with a gas mixture of 3.5 % O₂, 10 % CO₂ and 86.5 % N₂.

Preparation of *L. monocytogenes* immunogens and immunization of mice. For preparation of insoluble antigens of *L. monocytogenes*, cells were harvested from 200 ml overnight culture ($\sim 1.8 \times 10^{11}$ cells) by centrifugation at 10 000 g for 10 min at 4 °C, washed three times with PBS and killed by resuspending in 30 ml 0.3 % formalin solution in PBS for 24 h at room temperature. Cell pellets were washed with PBS, resuspended in 30 ml PBS containing 45 µg protease inhibitor cocktail ml⁻¹ (Sigma) and disrupted by passing through a French Press at 1500 p.s.i. (10 350 kPa). The homogenate was treated with DNase I (100 µg ml⁻¹) and RNase A (100 µg ml⁻¹) at 37 °C for 30 min and then spun at 100 000 g for 80 min at 4 °C. The pellets, designated insoluble antigens, were resuspended in 3 ml PBS and stored at -20 °C until use. For preparation of formalin-killed *L. monocytogenes*, cells were harvested from 40 ml overnight culture, washed as above and adjusted to a density of 1×10^9 cells ml⁻¹ in PBS containing 0.3 % formalin. After incubation at room temperature for 24 h, cells were centrifuged at 10 000 g for 10 min at 4 °C, washed with PBS and resuspended in an equal volume of PBS. Formalin-killed *L. monocytogenes* cells were stored at -20 °C until use.

Each of three ND4 mice, after their pre-immune sera were collected, was immunized subcutaneously with insoluble antigens derived from 6×10^9 cells in 0.1 ml PBS emulsified with an equal volume of complete Freund's adjuvant at day 0. Booster injections with the same amount of immunogens emulsified with incomplete Freund's adjuvant were given at days 14 and 28. After resting for at least 4 weeks, mice were given a final intravenous injection with the insoluble antigens (in 100 µl PBS) derived from 6×10^9 cells and sacrificed within 1 week for fusions. For immunization with formalin-killed cells, each of three ND4 mice was injected intraperitoneally with 2.5×10^8 cells in 0.25 ml PBS at days 0, 14 and 28. Mice were given a final intravenous booster injection with 2.5×10^7 cells in 0.15 ml PBS after resting for at least 4 weeks and sacrificed for fusion as above.

Preparation of bacterial ELISA antigens. *L. monocytogenes* (9×10^{11} cells) from 1 litre overnight culture were killed with formalin, washed and disrupted in 15 ml PBS containing a protease inhibitor cocktail (45 µg ml⁻¹) using a French Press. The homogenate, designated whole-cell (WC) antigens, was treated with DNase I and RNase A as above. The insoluble cellular (IC) antigens of *L. monocytogenes* were also prepared from 1 litre overnight culture (9×10^{11} cells) and resuspended in 5 ml PBS. WC antigens from other bacteria were prepared essentially as above: *L. innocua* WC antigens from 500 ml overnight culture (3.3×10^{11} cells) were resuspended in 10 ml PBS; *E. coli* O157:H7 WC antigens from 200 ml overnight culture (2.4×10^{11} cells) were resuspended in 15 ml PBS; *S.*

Typhimurium WC antigens from 50 ml overnight culture (3.3×10^{11} cells) were resuspended in 10 ml PBS; and *C. jejuni* WC antigens from a 200 ml cell suspension ($OD_{600} = 1.35$) were harvested by washing 25 100 mm plates with PBS and resuspended in 10 ml PBS. All antigen preparations were stored at -20°C until use.

Production of murine mAbs. Spleen cells, harvested from two ND4 mice receiving formalin-killed bacteria and from one ND4 mouse immunized with the insoluble antigens, were fused with Sp2/0-Ag14 myeloma cells (Shulman *et al.*, 1978), essentially according to established procedures (Zola, 1987). Culture supernatants from hybridoma cells were screened for *Listeria*-reactive mAbs by ELISA using WC antigens or the IC antigens derived from *L. monocytogenes* serotype 4b. A Nunc microwell plate (VWR) was coated with the antigens at a pre-determined dilution (1:400) in 60 mM carbonate buffer (pH 9.6; 100 μl per well), incubated at room temperature overnight and then frozen at -20°C until use. After the plates had been thawed and washed with PBS-T (0.05% Tween 20 in PBS, pH 7.2), hybridoma culture supernatants (100 μl per well) were added to react with the solid-phase antigens for 1 h at room temperature. Mouse anti-*Listeria* immune sera at pre-determined dilutions and 10% (v/v) fetal calf serum in the hybridoma growth medium were included as controls. Plates were washed with PBS-T, followed by incubation with HRP-conjugated goat anti-mouse IgG (1:4000 dilution in PBS-T, 100 μl per well) for 1 h at room temperature. After a final wash with PBS-T, substrate solution containing 1 mM ABTS and 0.015% H_2O_2 in 50 mM sodium citrate buffer (pH 4.5) was added to each well (100 μl per well). Following incubation with moderate shaking for 10 min at room temperature, A_{414} was determined on a Labsystems Multiskan Bichromatic plate photometer. Hybridomas cell lines secreting reactive mAbs were identified by using an empirical A_{414} cut-off of 0.3 under the conditions used.

Isotyping of murine immunoglobulins. The subclasses of immunoglobulins (Igs) secreted by the hybridoma cell lines were determined according to the standard operation procedures MC-PR021.02 and MC-PR022.03 (Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield, Ottawa, Ontario, Canada). Briefly, 20 μl hybridoma tissue culture fluid, pre-diluted 1:2 with Tris-buffered saline (0.01 M Tris/HCl pH 8.0, 0.15 M NaCl) containing 0.05% (v/v) Tween and 0.02% (w/v) NaN_3 , was incubated for 15 min at room temperature with 0.7–0.9 μm polystyrene beads (IDEXX Laboratories) that had been pre-coated with goat anti-mouse Ig (IgM + IgG + IgA, H + L chain-specific) (Southern Biotechnology) in the wells of Fluoricon assay plates (IDEXX Laboratories). After washing, bound murine Ig was detected with FITC-conjugated goat anti-mouse H + L chain-specific antibodies (Southern Biotechnology) using a Baxter fluorescence concentration analyser (IDEXX Laboratories). Negative and positive tissue-culture fluid controls were performed in parallel with the tested hybridoma tissue fluids.

Analysis of cross-reactivity of anti-*L. monocytogenes* mAbs with other micro-organisms. *L. monocytogenes*-reactive mAbs were assessed for cross-reactivity with *L. innocua*, *E. coli* O157:H7, *S. Typhimurium* and *C. jejuni* by indirect ELISA essentially as described above using the respective WC antigens.

Electrophoresis and Western blotting. SDS-PAGE was performed by the method described by Laemmli (1970), using a 4% stacking gel and a 12% resolving gel in a minigel apparatus (Bio-Rad). The protein samples for SDS-PAGE were prepared as follows. *L. monocytogenes* serotype 4b (overnight culture) was subcultured at a 1:100 dilution in 500 ml growth broth at 37°C for 4 h. Bacterial cells, equivalent to a 50 ml culture with an OD_{620} of 0.5, were harvested by centrifugation (8000 g, 20 min) and suspended in 1 ml 0.5 M Tris/HCl (pH 6.8) containing 10% SDS and 1 mM PMSF.

The cell suspension was sonicated ten times for 30 s each with 1 min intervals. Lysed cells were pre-incubated with an equal volume of $2 \times$ SDS/ β -mercaptoethanol sample buffer at 100°C for 10 min and then loaded (20 μl) into each well of the gels. Following electrophoresis at 200 V, proteins were either stained with Coomassie blue or electroblotted onto nitrocellulose membrane using a Bio-Rad Trans-Blot SD semi-dry transfer cell according to the manufacturer's manual. The membrane, which had been stained with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid to ensure the presence of proteins, was blocked with 3% skimmed milk in PBS containing 0.05% Tween 20 and 0.2% Triton X-100 (PBS-TT) for at least 1 h and incubated at room temperature with specific mAbs at a pre-determined dilution in PBS-TT containing 3% BSA. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and a 4-chloro-1-naphthol/ H_2O_2 substrate kit (Bio-Rad) according to the manufacturer's instructions.

Immunofluorescent staining. Binding of mAbs to the cell surface of *L. monocytogenes* was detected by immunofluorescent staining. Bacterial cells (2×10^8) from an overnight culture were spun at 13 000 r.p.m. in a microfuge for 2 min to remove the culture supernatant. Cell pellets were resuspended in 500 μl PBS containing 5% BSA, gently rocked for 1 h and then incubated for 1 h with 500 μl anti-*Listeria* mAb (tissue-culture fluid) at a dilution of 1:50 in PBS containing 5% BSA. After washing twice with 500 μl PBS, pellets were incubated for 1 h with 250 μl FITC-conjugated goat anti-mouse IgG (H + L chain) at a 1:100 dilution in PBS containing 5% BSA. Cell pellets were washed three times with PBS and resuspended in 50 μl PBS. One drop of the cell suspension was deposited onto a microscope slide, covered with a coverslip and sealed with Cytoseal 60 (VWR). Cells were viewed under an epifluorescence microscope.

Immunogold labelling for electron microscopy. *L. monocytogenes* serotype 4b (3×10^8 cells) from overnight culture were centrifuged at 13 000 r.p.m. for 2 min, washed twice with PBS and blocked with 500 μl PBS containing 3% BSA for 30 min at room temperature with a slow rotation. After washing with PBS, cells were incubated with a specific mAb at a 1:25 dilution in 0.5 ml PBS containing 3% BSA at room temperature for 1 h. Cells were then washed three times with PBS and incubated with 12 nm colloidal gold-conjugated goat anti-mouse IgG (H + L chain) at a 1:30 dilution in 0.3 ml PBS containing 3% BSA for 30 min as above. After three final washings with PBS followed by H_2O , cells were resuspended in 50 μl H_2O , adsorbed onto a carbon-coated nickel grid and stained with 0.5% uranyl acetate (0.22 μm filtered) for 5 s. Bacteria labelled with gold particles were viewed with a transmission electron microscope.

RESULTS

Production of mAbs to *L. monocytogenes*

The hybridomas resulting from three fusions, two with mice immunized with *L. monocytogenes* cells and one with a mouse inoculated with the IC antigen preparation, were screened for reactivity of secreted antibodies with *L. monocytogenes* by ELISA (Fig. 1a, b). A total of 35 mAbs (Table 1) was selected for further characterization on the basis of the mAbs ability to recognize both WC and IC antigens of *L. monocytogenes*. Of these 35 mAbs, 13 belonged to Ig subclass G1 (IgG1), 15 were IgG2a and 7 were IgM.

Fig. 1. Analysis of reactivity of mAbs raised against *L. monocytogenes* by ELISA. ELISA plates were coated with WC antigens of *L. monocytogenes* serotype 4b (Lm WC Ag) (a), IC antigens of *L. monocytogenes* serotype 4b (Lm IC Ag) (b) and WC antigens of *E. coli* O157:H7 (Ec WC Ag) (c), *S. Typhimurium* (S WC Ag) (d), *C. jejuni* (Cj WC Ag) (e) and *L. innocua* (Li WC Ag) (f) at a dilution of 1:500 (solid bars) or 1:1000 (open bars).

Western blot analysis of mAb reactivity

Analysis of the total cellular antigens of *L. monocytogenes* using Western blotting (Fig. 2) revealed that 20 out of the 35 mAbs were capable of detecting protein bands of various sizes, while 15 failed to react with any protein band on the blots. M2360, M2361, M2362 and M2389 reacted weakly with a protein band with an apparent molecular mass of 20 kDa; M2363 recognized a protein band of 87 kDa; M2369

detected a protein of 88 kDa and also a smaller band of 56 kDa; M2370, M2371, M2373 and M2374 detected a protein band of approximately 88 kDa; M2375 and M2392 reacted with a 77 kDa protein band; M2382 recognized a protein band with an apparent molecular mass of 75 kDa; M2384 detected a protein of approximately 62 kDa; M2385, M2386, M2387, M2394 and M2395 reacted with a protein band of 35 kDa; and M2393 reacted weakly with a protein band of 36 kDa.

Table 1. mAb reactivity with *E. coli* O157:H7 (Ec), *S. Typhimurium* DT104 (S), *C. jejuni* (Cj) and *L. innocua* (Li) as determined by indirect ELISA

All mAbs listed in the table showed the same reactions with WC and IC antigens of *L. monocytogenes* serotype 4b. IC antigens from the other four bacteria were not tested. —, Negative result, $A_{414} < 0.1$; +, positive result, $A_{414} > 0.3$; w+, weakly positive result, $A_{414} < 0.3$.

mAb	Isotype	Reactivity with ELISA antigens			
		Ec WC	S WC	Cj WC	Li WC
M2360	G2a	—	—	—	w+
M2361	G2a	—	—	—	w+
M2362	G2a	—	—	—	w+
M2363	G1	—	—	—	w+
M2364	G2a	—	—	—	w+
M2365	M	—	—	—	w+
M2366	G2a	—	—	—	+
M2367	M	—	—	—	w+
M2368	G1	—	—	—	w+
M2369	G2a	—	—	—	+
M2370	G2a	—	—	—	+
M2371	G2a	—	—	—	w+
M2372	M	+	w+	+	+
M2373	G2a	—	—	—	+
M2374	G2a	—	—	—	+
M2375	G2a	—	—	—	+
M2376	G2a	w+	—	—	+
M2377	G2a	w+	—	—	+
M2378	G1	+	w+	+	+
M2379	G1	w+	—	—	w+
M2380	M	+	—	+	w+
M2381	G1	—	—	—	w+
M2382	G1	—	—	—	w+
M2384	G1	—	—	—	w+
M2385	G2a	—	—	—	w+
M2386	G1	—	—	—	—
M2387	G1	—	—	—	—
M2388	M	+	w+	w+	+
M2389	G1	—	—	—	+
M2390	M	+	w+	w+	+
M2391	G1	—	—	—	—
M2392	G1	—	—	—	+
M2393	M	+	—	—	w+
M2394	G2a	—	—	—	—
M2395	G1	—	—	—	w+

Detection of *L. monocytogenes* cell-surface-exposed antigens by mAbs

Live *L. monocytogenes* were probed with each of the 35 mAbs followed by immunofluorescent staining. Only M2365 and M2367 recognized antigens present on the cell surface (Fig. 3). Staining by these two mAbs was specific, as no staining was observed with a control mAb M2165 (data not shown). All other mAbs, exemplified in Fig. 3 by M2387,

failed to stain live bacteria, indicating that the epitopes recognized by these antibodies are not accessible on the cell surface. Immunofluorescent microscopy also revealed that neither M2365 nor M2367 bound to the cell surface of live *E. coli* O157:H7, *S. Typhimurium* or *C. jejuni* (data not shown). Consistent results were obtained for all of the mAbs with immunogold labelling of *L. monocytogenes*. Transmission electron microscopy showed immunogold particles on the surface of the bacterium probed with M2365 and M2367 (Fig. 4). Probing with the control mAb M2165 (Fig. 4) and all of the other mAbs directed against *L. monocytogenes* (data not shown) did not result in the presence of gold particles on the surface of bacterial cells.

Cross-reaction of anti-*L. monocytogenes* mAbs with other selected bacteria

All 35 mAbs that were shown to react with the WC and IC antigens of *L. monocytogenes* (Fig. 1a, b) were tested for reactivity with other food-borne pathogens represented by *E. coli* O157:H7, *S. Typhimurium*, *C. jejuni* and the non-pathogenic bacterium *L. innocua* by ELISA (Fig. 1c–f). By using a cut-off A_{414} value of 0.1, which was approximately ten times the value obtained with the control antibody, nine mAbs (M2372, M2376, M2377, M2378, M2379, M2380, M2388, M2390 and M2393) were found to react with *E. coli* O157:H7 (Fig. 1c), four (M2372, M2378, M2388 and M2390) with *S. Typhimurium* (Fig. 1d) and five (M2372, M2378, M2380, M2388 and M2390) with *C. jejuni* (Fig. 1e). All mAbs except for M2366, M2369, M2370, M2372, M2373, M2374, M2375, M2376, M2377, M2378, M2388, M2389, M2390 and M2392 exhibited a weak or no reaction with *L. innocua*, with A_{414} values of < 0.3 (Fig. 1f). The two cell-surface-binding antibodies M2365 and M2367 were tested further by ELISA for interaction with WC antigens derived from *L. monocytogenes* serotypes 1/2a (LI0527), 1/2b (LI0586) and 3a (LI0508) and *Listeria* species *Listeria grayi* (ATCC 19120), *Listeria ivanovii* (ATCC 19119) and *Listeria seeligeri* (PHB24) and exhibited no binding to these antigens, with A_{414} values of ≤ 0.1 .

DISCUSSION

The aim of this study was to develop mAbs directed against *L. monocytogenes*. A total of 35 mAbs reacting with *L. monocytogenes* was identified by screening the hybridomas produced from three fusions. Binding activities of these mAbs were studied using techniques such as ELISA, Western blotting, epifluorescence microscopy and immunogold electron microscopy. The majority of mAbs identified were IgG1 or IgG2a, with a few being IgM. Of these 35 mAbs, only 20 rapidly detected protein bands in Western blots of total cellular proteins, although 5 exhibited weak immunological reactions, indicating that the epitopes defined by these antibodies are linear. The other 15 mAbs exhibiting no reactivity in Western blots probably recognized conformational epitopes, which may have been altered under the denaturing conditions used in SDS-PAGE; alternatively, the

Fig. 2. Western blot analysis of total cell proteins of *L. monocytogenes* serotype 4b. Total proteins derived from 8×10^8 bacterial cells were probed with each of the 35 mAbs following SDS-PAGE and electroblotting onto a nitrocellulose membrane. Binding was detected by reaction with HRP-conjugated goat anti-mouse IgG (H+L chain). Protein standards (Std) with their molecular masses in kDa are shown on the left of the blots. The blank unlabelled lanes in the middle and bottom panels on the right show the results with some of the unreactive mAbs used in the other four panels.

epitopes recognized by them may have been of a non-protein nature and not transferred efficiently onto the nitrocellulose membranes. This observation is not unusual for anti-*Listeria* mAbs, as Kathariou *et al.* (1994) and Torensma *et al.* (1993) reported that some of their mAbs failed to detect protein bands in Western blots of total cellular proteins. With the exception of M2369, which reacted with a protein of 88 kDa and a smaller protein with an apparent molecular mass of 56 kDa, all other mAbs that reacted with *L. monocytogenes* antigens on Western blots detected a single protein band with apparent molecular masses of 20 kDa (four mAbs), 35 kDa (five mAbs), 36 kDa (one mAb), 62 kDa (one mAb), 75 kDa (one mAb), 77 kDa (two mAbs) and 87–88 kDa (six mAbs). In contrast, slightly complicated immunoreaction patterns for mAbs directed

against *Listeria* were demonstrated by Western blotting in several previous studies, detecting two major antigens of 38 and 41 kDa (Loiseau *et al.*, 1995); a protein smear of 40–60 kDa, several vague bands of ~20 kDa and double bands at 90 and 35 kDa (Torensma *et al.*, 1993), multiple bands of 76, 66, 56 and 52 kDa (Bhunia *et al.*, 1991), a protein band of 66 kDa and double bands of 43 and 94–97 kDa (Bhunia & Johnson, 1992), a 31 kDa flagellin (Kathariou *et al.*, 1994), an 18.5 kDa protein (Siragusa & Johnson, 1990) and a 104 kDa cell-surface protein (Pandiripally *et al.*, 1999). Thus, the mAbs reported here with reactivity on Western blots appeared to recognize linear epitopes localized on proteins that were different in size from the mAb-reactive proteins described by others (Siragusa & Johnson, 1990; Bhunia *et al.*, 1991; Bhunia & Johnson, 1992; Torensma

Fig. 3. Immunofluorescent staining of *L. monocytogenes* serotype 4b. Bacterial cells (2×10^8) were probed with each of the 35 mAbs. Binding was detected by reaction with FITC-conjugated goat anti-mouse IgG (H+L chain). Cells were visualized with a fluorescence microscope. Fluorescent images (left) and phase-contrast images (right) of bacterial cells in the same field are shown for staining with three selected mAbs (M2365, M2367 and M2387).

et al., 1993; Kathariou *et al.*, 1994; Loiseau *et al.*, 1995; Pandiripally *et al.*, 1999). Thus, they are likely to be unique immunogenic proteins.

Specific binding to the cell surface with minimal or no cross-reaction with other micro-organisms is a desired feature for a mAb to be useful in the detection of *L. monocytogenes*. Of the 35 mAbs described here, only M2365 and M2367 recognized cell-surface antigens, as demonstrated by epifluorescence microscopy and immunogold electron microscopy. These two mAbs possibly recognized a conformational epitope, as they did not produce reactivity in Western blot analysis. These two antibodies did not cross-react with *E. coli* O157:H7, *C. jejuni* and *S. Typhimurium*, and showed a very weak reaction with total *L. innocua* cellular antigens in ELISA. M2365 and M2367 also failed to recognize the surface antigens of *E. coli* O157:H7, *C. jejuni* and *S. Typhimurium*,

as revealed by immunofluorescent staining. In addition, M2365 and M2367 showed no reaction with whole-cell antigens from *L. monocytogenes* serotypes 1/2a (LI0527), 1/2b (LI0586) and 3a (LI0508) and the *Listeria* species *L. grayi* (ATCC 19120), *L. ivanovii* (ATCC 19119) and *L. seeligeri* (PHB24) in ELISA. The lack of cross-reaction of M2365 and M2367 with a limited number of *L. monocytogenes* serotypes and other bacterial species suggests that M2365 and M2367 have potential for use in the immunocapture of live *L. monocytogenes* for rapid detection in food or other test samples, and for clinical applications such as the detection of the bacterium in infected tissues or cerebrospinal fluid. The use of mAbs as reagents for the development of rapid methods of laboratory diagnosis to detect *Listeria* from food or clinical samples has been described in other studies (McLauchlin *et al.*, 1988, 1989; McLauchlin & Taylor, 1989). The present study has added new mAbs to the

Fig. 4. Detection of *L. monocytogenes* surface antigens by immunogold labelling with mAbs. Bacterial cells (3×10^8) were probed with each of the 35 mAbs followed by detection with 12 nm gold particle-conjugated goat anti-mouse IgG (H+L chain). Bacterial cells were visualized with a transmission electron microscope at a magnification of $\times 30\,000$. Images of immunogold labelling with two mAbs (M2365, M2367) and one control mAb (M2165) are shown. Bar, 0.6 μm .

diagnostically important antibody list. Those mAbs showing no binding to the surface antigens but demonstrating reactivity with WC lysate and IC antigens in ELISA, as described in this study, may recognize epitopes that are not on the surface but which rapidly become exposed and accessible as a result of the mechanical disruption of intact *L. monocytogenes*. Of the 35 mAbs directed against *L. monocytogenes*, only a few exhibited a significant cross-reaction with *E. coli* O157:H7, *C. jejuni* or *S. Typhimurium*. As expected, there were more mAbs that showed a significant cross-reaction with the antigens of *L. innocua*. Four mAbs (M2372, M2378, M2388 and M2390) appeared to

recognize a common conformational epitope among the bacterial species tested, as indicated by ELISA and Western blotting. Four mAbs (M2386, M2391, M2387 and M2394) showed no reaction with *E. coli* O157:H7, *C. jejuni*, *S. Typhimurium* and *L. innocua* in ELISA, and thus are highly specific for *L. monocytogenes*. Two mAbs, M2386 and M2394, recognized a linear epitope on proteins with similar molecular masses of 35 kDa.

In summary, we have successfully developed a panel of 35 mAbs directed against *L. monocytogenes*. Some of the mAbs described here may be useful as reagents for the specific detection of the bacterium in food or clinical samples, and as potential probes for serotyping the 4b strains and studying their surface-antigen composition.

ACKNOWLEDGEMENTS

We acknowledge D. Franks for assistance with fluorescent microscopy, B. Phipps-Todd for help with electron microscopy, J. Devenish for assistance in *C. jejuni* culture and L. Wang for valuable discussion and suggestions about immunofluorescent staining and immunogold labelling procedures. We are also grateful for support from the Small Animal Colony and the Monoclonal Antibody Unit (Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield) for mouse immunization and hybridoma fusion.

REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (2005). *Current Protocols in Molecular Biology*. New York: Wiley.
- Bhunja, A. K. & Johnson, M. G. (1992). Monoclonal antibody specific for *Listeria monocytogenes* associated with a 66-kilodalton cell surface antigen. *Appl Environ Microbiol* **58**, 1924–1929.
- Bhunja, A. K., Ball, P. H., Fuad, A. T., Kurz, B. W., Emerson, J. W. & Johnson, M. G. (1991). Development and characterization of a monoclonal antibody specific for *Listeria monocytogenes* and *Listeria innocua*. *Infect Immun* **59**, 3176–3184.
- Donnelly, C. W. (2001). *Listeria monocytogenes*: a continuing challenge. *Nutr Rev* **59**, 183–194.
- Farber, J. M. & Speirs, J. I. (1987). Potential use of continuous cell lines to distinguish between pathogenic and nonpathogenic *Listeria* spp. *J Clin Microbiol* **25**, 1463–1466.
- Kathariou, S., Mizumoto, C., Allen, R. D., Fok, A. K. & Benedict, A. A. (1994). Monoclonal antibodies with a high degree of specificity for *Listeria monocytogenes* serotype 4b. *Appl Environ Microbiol* **60**, 3548–3552.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lin, M., Trottier, E. & Mallory, M. (2005). Enzyme-linked immunosorbent assay based on a chimeric antigen bearing the antigenic regions of structural proteins E^{rms} and E2 for the serodiagnosis of classical swine fever virus infection. *Clin Diagn Lab Immunol* **12**, 877–881.
- Loiseau, O., Cottin, J., Robert, R., Tronchin, G., Mahaza, C. & Senet, J. M. (1995). Development and characterization of monoclonal antibodies specific for the genus *Listeria*. *FEMS Immunol Med Microbiol* **11**, 219–230.
- Lorber, B. (1997). Listeriosis. *Clin Infect Dis* **24**, 1–9.
- McLauchlin, J. & Taylor, A. G. (1989). The use of monoclonal antibodies in the characterization and purification of cell surface

antigens of *Listeria monocytogenes* serogroup 4. *Acta Microbiol Hung* 36, 459–465.

McLauchlin, J., Black, A., Green, H. T., Nash, J. Q. & Taylor, A. G. (1988). Monoclonal antibodies show *Listeria monocytogenes* in necropsy tissue samples. *J Clin Pathol* 41, 983–988.

McLauchlin, J., Samuel, D. & Taylor, A. G. (1989). The use of monoclonal antibodies to demonstrate *Listeria monocytogenes* in post mortem tissue using a direct immunofluorescence technique, and to detect a soluble antigen in CSF supernatants using an ELISA. *Acta Microbiol Hung* 36, 303–308.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerg Infect Dis* 5, 607–625.

Norton, D. M. (2002). Polymerase chain reaction-based methods for detection of *Listeria monocytogenes*: toward real-time screening for food and environmental samples. *J AOAC Int* 85, 505–515.

Pandiripally, V. K., Westbrook, D. G., Sunki, G. R. & Bhunia, A. K. (1999). Surface protein p104 is involved in adhesion of *Listeria monocytogenes* to human intestinal cell line, Caco-2. *J Med Microbiol* 48, 117–124.

Ralovich, B. (1993). Detection and epidemiological typing of *Listeria* strains – diagnostic methods for *Listeria* infections (a review). *Acta Microbiol Hung* 40, 3–38.

Rocourt, J. & Bille, J. (1997). Foodborne listeriosis. *World Health Stat Q* 50, 67–73.

Schlech, W. F., III (1993). An animal model of foodborne *Listeria monocytogenes* virulence: effect of alterations in local and systemic immunity on invasive infection. *Clin Invest Med* 16, 219–225.

Schlech, W. F. III (2000). Foodborne listeriosis. *Clin Infect Dis* 31, 770–775.

Shulman, M., Wilde, C. D. & Köhler, G. (1978). A better cell line for making hybridomas secreting specific antibodies. *Nature* 276, 269–270.

Siragusa, G. R. & Johnson, M. G. (1990). Monoclonal antibody specific for *Listeria monocytogenes*, *Listeria innocua*, and *Listeria welshimeri*. *Appl Environ Microbiol* 56, 1897–1904.

Torensma, R., Visser, M. J., Aarsman, C. J., Poppelier, M. J., Fluit, A. C. & Verhoef, J. (1993). Monoclonal antibodies that react with live *Listeria* spp. *Appl Environ Microbiol* 59, 2713–2716.

Yu, W. (2004). *The gene products of Listeria monocytogenes induced specifically during rabbit infection*. MSc thesis, University of Ottawa, Ottawa, Canada.

Ziegler, H. K. & Orlin, C. A. (1984). Analysis of *Listeria monocytogenes* antigens with monoclonal antibodies. *Clin Invest Med* 7, 239–242.

Zola, H. (1987). *Monoclonal Antibodies: a Manual of Techniques*. Boca Raton, FL: CRC Press.