Monoclonal Antibodies with a High Degree of Specificity for 
Listeria monocytogenes Serotype 4b

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Strains of Listeria monocytogenes serotype 4b account for a large fraction of sporadic listeriosis cases, as well as all major food-borne epidemics attributed to this pathogen. We have identified a set of three monoclonal antibodies which showed a high degree of specificity for strains of L. monocytogenes serotype 4b. Two of these antibodies (c74.33 and c74.180, isotypes immunoglobulin M [IgM] and IgG3, respectively) recognized all serotype 4b strains, whereas antibody c74.22 (isotype IgG1) failed to recognize certain epidemic-associated strains. The corresponding antigens were located on the surface of the bacteria and were expressed following bacterial growth in different media and over a wide range of temperatures (4, 22, and 37°C). Heating L. monocytogenes cells at 80, 90, or 100°C abolished reactivity for c74.22 but not for c74.33 MAb. These MAbs were negative for all of the non-Listeria strains tested, including representatives of several gram-negative and gram-positive species. The surface antigen recognized by c74.22 appeared to be associated with the ability of the bacteria to enter (invade) mammalian cells in culture.

Listeria monocytogenes is a gram-positive bacterium that is widespread in the environment and capable of causing serious disease (listeriosis) in humans and animals (15, 27, 28). At risk are especially newborns, the elderly, and immunocompromised individuals. The bacteria are capable of growth at low temperatures and can thus cause serious contamination of cold-stored foods. Recently, several epidemics of listeriosis have been attributed to contamination of food, especially dairy products, with L. monocytogenes (27).

Several serotypes of listeriae have been identified by using the antigenic scheme of Seeliger and Hohn (29). Even though numerous serotypes of L. monocytogenes are isolated from foods and from the environment (10, 17, 26), only a few (1/2a, 1/2b, and 4b) are frequently seen in clinically derived strains. Serotype 4b strains account for ca. 40% of sporadic listeriosis cases. Furthermore, strains implicated in large food-related epidemics of listeriosis have invariably been of serotype 4b (27). It thus seems important to monitor the occurrence of this serotype in foods. Serotyping of listeriae by conventional means, however, is not trivial, as it requires highly specific antisera and dedicated reference laboratory facilities.

Our understanding of the cell surface of listeriae is limited (12, 25), and the chemical and genetic characteristics underlying the serotypic designations of these bacteria remain unclear. Such designations reflect both somatic and flagellar antigens of the microorganism (29). Recent data suggest that strains of some serotypes (1/2a, 3a, and 1/2c) are genetically quite distinct from strains of serotypes 1/2b and 4b (5, 6, 6a, 16, 21). Furthermore, DNA sequence data on specific genes (hly and flaA, encoding hemolysin and flagellin, respectively) suggest that polymorphisms at the DNA sequence level differentiate strains of serotypes 1/2b and 4b from those of serotypes 1/2a, 3a, and 1/2c (8, 24).

We have initiated a series of investigations aimed at characterization of the genetic basis of serotype 4b-specific antigens of L. monocytogenes. As a first step in studying such antigens, we have generated monoclonal antibodies (MAbs) with a high degree of specificity for strains of this serotype. In this report, we describe these antibodies. Furthermore, we show that certain of the epidemic-associated strains of L. monocytogenes have unique patterns of reactivity with these MAbs which readily differentiate them from other strains of the same serotype.

MATERIALS AND METHODS

Bacteria and growth media. Unless otherwise indicated, Listeria strains were grown for 36 h on Trypticase soy agar medium (Difco, Detroit, Mich.) supplemented with 0.7% yeast extract (Difco) at room temperature (22°C). Liquid cultures were grown in the same medium without agar or in brain heart infusion broth (Difco). When grown at 37°C, both liquid and agar cultures were incubated in a 37°C incubator for 18 h. For growth at 4°C, 30 µL from an overnight culture grown at 37°C was spread on the surface of an agar plate and kept refrigerated until the bacteria grew sufficiently (usually 2 to 4 weeks).

Production of MAbs and enzyme-linked immunosorbent assay (ELISA). The original antigen was prepared as follows. L. monocytogenes 4b1, a streptomycin-resistant derivative of strain NCTC 10527, was used for inoculum preparation. The growth from a 36-h-old confluent agar culture of the bacteria at 22°C was resuspended in phosphate-buffered saline (PBS) containing 0.5% (vol/vol) formalin and allowed to stand at 22°C for 24 h. Conventional methodology was used for immunization of the mice and generation of the hybridomas (1). Whole-cell ELISA was done as previously described (2). ELISA results were recorded as negative if the absorbance values were in the same range as those for the reagent controls (less than 0.1). Typical absorbance values for positive ELISA results were in the range of 0.8 to 1.2.

Immungold labeling, immunofluorescence, and Western blots (immunoblots). For immungold labeling of the bacteria, drops of a washed and fixed bacterial suspension were placed on a Formvar-supported grid and allowed to stand. After 5 min, the excess fluid was removed and the grid with its attached cells was floated for 10 min each time on 2 drops of Tris-
TABLE 1. Reactivity of *Listeria* strains with MAbs c74.22 and c74.33 as determined by ELISA

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. screened</th>
<th>No. positive with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c74.22</td>
<td>c74.33</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> serotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2a</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>1/2b</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>4b</td>
<td>25</td>
<td>23* 25</td>
</tr>
<tr>
<td>1/2c, 3c</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>4a/b</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4a</td>
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</tr>
<tr>
<td>4c</td>
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</tr>
<tr>
<td>4d</td>
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<td>1</td>
</tr>
<tr>
<td>4e</td>
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<td>1</td>
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<tr>
<td>3a</td>
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<tr>
<td>3b</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td><em>L. ivanovii</em></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>L. seeligeri</em></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td><em>L. welschimeri</em></td>
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<td>0</td>
</tr>
<tr>
<td><em>L. grayi</em></td>
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<td>0</td>
</tr>
<tr>
<td>Atypical <em>Listeria</em> serotypes b</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6a</td>
<td>0</td>
</tr>
</tbody>
</table>

* The nonreactive strains included the two epidemic-associated strains mentioned in the text. Transposon mutants and other variants were not included. 
* b *Listeria* strains which are rhombose negative and have uncertain taxonomic designations.

buffered saline containing 1% bovine serum albumin (BSA). Following 30 min of incubation in undiluted hybridoma supernatant, the grids were washed for 30 min in Tris-buffered saline with 0.1% BSA. After 30 min of incubation in goat anti-mouse immunoglobulin G conjugated to 18-nm colloidal gold particles, the grids were again washed for 30 min in Tris-buffered saline with 0.1% BSA and for 5 min in distilled water. The final incubation was on 1% uranyl acetate. Excess uranyl acetate was blotted, and the grids were air dried. The cells were examined in a Zeiss EM10 electron microscope operated at 80 kV.

For immunofluorescence, overnight cultures of bacteria were treated with 0.5% formalin. The cells were washed four times with PBS and resuspended in PBS at an A<sub>600</sub> of 0.05; 30 μl was placed on a clean glass slide, air dried, and fixed in absolute methanol for 15 min at −20°C. The slide was washed with PBS, and a drop of a diluted (1:400) MAb was added. After 20 min at room temperature, the slide was washed with PBS and 30 μl of a 1:20 dilution of rabbit anti-mouse immunoglobulin-fluorescein isothiocyanate (Sigma, St. Louis, Mo.) was added and allowed to stand for 20 min at room temperature. The slide was washed with PBS, air dried, mounted with 70% glycerol in PBS, and examined with a Nikon inverted epifluorescence microscope (DIAPHOT-TMD).

For Western blotting, total cellular proteins were prepared from 5-ml cultures in brain heart infusion broth or from agar-grown cultures (grown at either 22 or 37°C) and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (20). Extraction of surface proteins by SDS (1%, wt/vol) was done for 1 h at 37°C with agitation. Proteins were transferred to nitrocellulose (MSI; Fisher Scientific) by vacuum blotting (18) or by electrophoretic transfer with an electroblotting chamber from Bio-Rad. MAbs from both murine ascites and culture supernatants were used. Secondary horseradish peroxidase-conjugated anti-mouse antibodies were purchased from Promega Corp. (Madison, Wis.). An enhanced-chemiluminescence kit (Amersham) was used for signal detection.

**Heat treatments.** An Eppendorf microcentrifuge tube containing a formalinized suspension of strain 4b1 was placed in a water bath preset to the temperatures indicated elsewhere in the text. After the indicated time, the tube was removed and placed immediately on ice. After cooling, the bacteria were centrifuged and the cell pellet and the supernatant (containing heat-extractable antigens) were screened separately by ELISA as previously described (2).

**RESULTS**

**Identification of MAbs.** Screening of the hybridomas produced from one fusion with a small number of different *Listeria* strains suggested that two hybridomas (c74.22 and c74.33, of the immunoglobulin GI and M isotypes, respectively) reacted with serotype 4b *L. monocytogenes* but not with representatives of the other major serotypes of this species (1/2a and 1/2b). These MAbs were used in an extensive screening of additional strains. In an indirect ELISA, MAbs c74.22 and c74.33 gave consistently strong signals with strains of serotype 4b (typical absorbance values were 0.8 to 1.2) and, with only two exceptions (two strains of serotypes 4d and 4e, respectively), failed to react with strains of other *L. monocytogenes* serotypes (Table 1). A third hybridoma from the same fusion, c74.180 (isotype immunoglobulin G3), had the same reactivity pattern, although the strength of its reactivity in the ELISA was more variable than that of c74.22 and c74.33. In this presentation, we focus on the latter two MAbs.

We screened numerous strains of serotype 4b with these MAbs (Table 2). The serotype 4b group included strains designated epidemic which were implicated in documented common-source outbreaks of listeriosis (10, 27), as well as other strains (designated nonepidemic or sporadic) implicated in cases of listeriosis that were not part of a known common-source outbreak. Our screenings revealed that c74.33 reacted with
with all of the strains of this serotype. C74.22, on the other hand, reacted with all of the nonepidemic strains but failed to react with two of the four strains which were implicated in epidemics of food-borne listeriosis (Table 2). These MAbs c74.22-nonreactive strains were 15U and 18, implicated in the Nova Scotia epidemic and the 1983 Massachusetts epidemic (10, 27), respectively. Several transposon mutants of the strain used as the original immunogen (strain 4bl) were also screened for reactivity with the MAbs. MAb c74.22 did not react with one transposon mutant (4-44) of strain 4bl, which appeared to be deficient in the ability to invade 3T3 fibroblasts (23). Other transposon mutants of 4bl, including nonmotile and nonhemolytic mutants and filamentous (rough) mutants (19), maintained their reactivity with MAb c74.22 (Table 2). The only other serotype 4b strain which reacted with c74.33 but failed to react with c74.22 was strain F4244+, a hyperhemolytic variant of F4244 (22). Wild-type strain F4244 reacted with both c74.22 and c74.33. Surprisingly, strain F4244M, a nonhemolytic variant of F4244+, reacted with both antibodies (data not shown). These findings suggest that the serotype-specific antigen recognized by MAb c74.22 is missing or altered in the two epidemic-associated strains, as well as in the invasion-deficient mutant and hyperhemolytic strain F4244+.

**Immunogold labeling, immunofluorescence, and Western blots.** Immunogold labeling with MAb c74.22 confirmed that the corresponding antigen is on the surface of the bacteria (Fig. 1). The bacteria (strain 4bl) were also strongly and uniformly labeled with immunofluorescence by using MAb c74.22 (Fig. 2A) and, to a slightly lesser extent, with MAb c74.33 (Fig. 2B). Strains 15U and 18 and invasion-deficient mutant strain 4-44 were negative in immunofluorescence with MAb c74.22 but positive with c74.33, confirming the ELISA reactivity data (Table 2).

In Western blots of total cellular proteins (reduced by β-mercaptoethanol in the final sample buffer, as well as nonreduced) we were unable to consistently detect a band that was recognized by MAbs c74.22, c74.33, and c74.180. SDS extraction of surface proteins and use of the extracted material in Western blots with these MAbs also failed to identify a reacting band. Under similar conditions, Western blots of total cellular proteins and SDS-extracted proteins with another MAb which we have produced (c74.191, specific to *Listeria* flagellin) readily produced the expected signal, corresponding to a protein with an apparent molecular weight of 31,000 (data not shown).

**Sensitivity of c74.22 and c74.33 reactivity to heat.** Reactivity with c74.22 was abolished following extensive heat treatment (5 h at 80°C, 2 h at 90°C, or 40 min at 100°C). No reactivity with c74.22 was seen with the heat-treated cells or with the heat extract. In contrast, reactivity with c74.33 was not affected under these conditions (data not shown).

**Effect of bacterial growth conditions on expression of the putative serotype-specific antigens.** Several parameters, including growth media and temperature, were examined for effects on the expression of antigens recognized by c74.22 and c74.33. ELISA reactivity of the bacteria with the MAbs was maintained following growth on agar or in liquid media, in or on media based on Trypticase soy or brain heart infusion broth, and at different temperatures (4, 22, and 37°C). Furthermore, both formalin-fixed and live bacteria reacted with the MAbs. We observed, however, that growth of certain strains in liquid at 37°C occasionally decreased their reactivity with c74.22.

The lack of reactivity of certain epidemic strains and of noninvasive mutant 4-44 (Table 2) was unaffected by growth conditions, including growth of the bacteria at 4°C. Similarly, live and formalin-killed cells of these strains were negative with c74.22.

**Reactivity of other *Listeria* strains with MAbs.** A survey of other *L. monocytogenes* strains of various serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4b, 4ab, 4a, 4c, 4d, 4e, and 7) suggested that only strains of serotypes 4b, 4d, and 4e react with c74.22 and

**FIG. 1.** Immunogold labeling of *L. monocytogenes* 4b1 by MAb c74.22. Labeling was done as described in Materials and Methods. Panels A and B show different fields from the same grid. Bar, 0.5 μm.

**FIG. 2.** Immunofluorescence of *L. monocytogenes* 4b1 with MAbs c74.22 (A) and c74.33 (B). Immunofluorescence and photography were done as described in Materials and Methods.
c74.33 (Table 1). All of the serotype 4b *L. monocytogenes* strains which we screened reacted with MAbs c74.22 and c74.33. In fact, it was possible to detect strains the serotype of which had been mistakenly recorded as 4b by their lack of reactivity with this MAb. *Listeria* strains of other species did not react with these MAbs (absorbance values under 0.1, the same as those of the reagent controls), the only exception being three previously uncharacterized strains of *L. innocua* (Table 1). All other strains of *L. innocua*, as well as strains of *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi*, were negative with these antibodies (Table 1). Immunofluorescence labeling of the three reactive *L. innocua* strains with MAb c74.32 showed strong but somewhat patchy distribution of the label on the bacterial cell surface (data not shown). This was in contrast to the homogeneous immunofluorescent labeling of *L. monocytogenes* serotype 4b with c74.22 (Fig. 2A).

**Reactivity of non-*Listeria* strains with the serotype-specific MAbs.** A panel of bacterial strains representing several gram-negative and gram-positive species were screened with c74.22, c74.33, and c74.180 by using an ELISA. This panel of non-*Listeria* microorganisms included *Agrobacterium* sp. (2 strains), *Xanthomonas* sp. (12 strains), *Brucella abortus*, *B. melitensis*, *Yersinia enterocolitica*, *Pseudomonas syringae* (12 strains), several other plant-pathogenic pseudomonads (24 strains), *Enterobacter cloacae*, *Erwinia* sp. (4 strains), *Clavibacter* sp. (10 strains), *Bacillus firmus*, *B. megaterium*, *B. subtilis*, *B. brevis*, *B. cereus*, *Corynebacterium diphtheriae*, *C. pseudodiphtheriae*, *C. xerosis*, *Gaffkya tetragna*, *Micrococcus citreus*, *M. luteus*, *M. lysodeicticus*, *M. roseus*, *Streptococcus* sp. (groups A, B, D, and E), *Enterococcus faecalis*, *Staphylococcus aureus* (3 strains), and *S. epidermidis*. None of the non-*Listeria* organisms reacted with c74.22, c74.33, or c74.180 (absorbance values never exceeded 0.1).

**DISCUSSION**

Screenings of numerous *L. monocytogenes* strains with the MAbs described in this work revealed that these reagents reacted with antigens present on the surface of strains of serotype 4b. With the exception of the two strains of serotypes 4d and 4e, respectively, *L. monocytogenes* strains of other serotypes were negative for reactivity with these antibodies. We do not know whether this lack of reactivity in strains of the other serotypes is due to absence, antigenic divergence, or lack of exposure of the antigen(s) on the bacterial cell surface. Reactivity of serotype 4b strains with these MAbs clearly differentiates these strains from strains of serotype 1/2b, to which they have been shown to be genetically close (5, 6, 21).

On the other hand, these MAbs do not appear to recognize serogroup 4-specific surface antigens, since with the exception of the two strains of serotypes 4d and 4e, other serogroup 4 strains failed to be recognized by the MAbs (Table 1). Strains of serotypes 4d and 4e are not usually associated with clinical cases and are rare in culture collections of *L. monocytogenes*. Without additional strains and confirming serotypic data, we cannot exclude the possibility that these two strains are in reality of serotype 4b. It is of interest that on the basis of pulsed-field gel electrophoresis and cluster analysis data, these strains appeared to be closely related to serotype 4b strains and, in fact, could not be differentiated from the latter (6a).

Thus, the MAbs described in this work appear to recognize surface antigens characteristic of serotype 4b (and perhaps also of serotype 4d and 4e) strains. It must be emphasized that the biochemical characteristics underlying serotypic designations of *listeriae* remain unknown. These MAbs may be quite useful for identification of serotype-specific surface antigenic determinants.

The reactivity patterns of these antibodies indicate that they are different in terms of specificity from MAbs to surface antigens of *listeriae* described by other investigators, which are *Listeria* genus specific (7, 11) or specific for *L. monocytogenes* and one or more other *Listeria* species (3, 30, 31) or react preferentially (14) or exclusively (4) with *L. monocytogenes* strains of all serotypes.

Among the other *Listeria* strains which we screened (including 35 strains of *L. innocua*) we were able to identify only 3 *L. innocua* strains which reacted with the serotype-specific MAbs. We do not know the serotype of these strains, nor are we aware of any genetic or bacteriologic peculiarities of these bacteria. We can only speculate as to the biological significance of the reactivity of these strains with the MAbs. It is possible that they represent a genetically distinct subpopulation of *L. innocua* which shares surface antigenic determinants with *L. monocytogenes* serotype 4b. Alternatively, these *L. innocua* strains may have surface antigens which are biochemically distinct but antigenically cross-reactive with those recognized by c74.22 and c74.33.

With the exception of the two epidemic strains (Nova Scotia and Massachusetts strains) and variants 4-44 and F4244+, all of the strains we studied showed the same reactivity pattern with the two MAbs, being either positive with both or negative with both. The identification of strains which reacted with c74.33 but not c74.22 and the differential sensitivity of reactivity with these MAbs to heat treatment suggest that we are dealing with two distinct epitopes. We are currently pursuing the biochemical characterization of the antigens involved. Repeated attempts to consistently identify the antigens in Western blots were negative. The reasons for this are unclear. ELISA determinations suggest that the cellular component responsible for reactivity with the MAbs is tightly associated with the cell envelope and not readily extractable by standard methods (heat, SDS extraction). It is also possible that we are dealing with a nonproteinaceous antigen(s) which failed to transfer to nitrocellulose efficiently because of its large size and/or chemical properties.

We are especially interested in the biological significance of the putative serotype-specific antigen(s) for *L. monocytogenes*. Failure to detect any serotype 4b strains which lacked reactivity with c74.33 suggests that the corresponding antigen has an important role in these strains. On the other hand, the identification of natural isolates and transposon-induced variants which lacked reactivity with c74.22 (but were positive with c74.33) may help to elucidate the possible involvement of the corresponding surface antigen in the physiology and/or pathogenesis of the bacteria. The lack of MAb c74.22 reactivity in invasion-deficient mutant 4-44 suggests that the antigen that reacts with this antibody is associated with invasion of mammalian cells. The other surface antigen of *L. monocytogenes* which has been shown to be essential for invasion is internalin (13). Several aspects of 4-44, however, suggest that this mutant is phenotypically and genetically distinct from internalin mutants (9). In particular, internalin-deficient mutants failed to attach to mammalian cells as well as invade, whereas 4-44 was deficient in invasion but attached normally to cultured mammalian cells. In addition, the transposon insertion in 4-44 was not in the internalin gene family but in another, still unidentified locus (9).

The failure of the two epidemic strains to react with MAb 2c74.22 is intriguing, since all of the other wild-type serotype 4b strains reacted with this antibody. One may speculate that loss or alteration of the c74.22-reactive antigen is associated...
with the ability of certain L. monocytogenes strains to cause epidemic disease. Some isoyme typing data suggest that epidemic-associated strains of L. monocytogenes tend to have similar multilocus genotypes (21), indicating that they are genetically related. It must be emphasized, however, that we lack information on the microbiological and pathogenic features which differentiate strains implicated in sporadic disease from some or all of the strains implicated in epidemics. The serotype-specific antibodies may be useful tools for such investigations. They may, in addition, be useful as diagnostic reagents for L. monocytogenes serotype 4b, as probes for the surface antigenic composition of these strains, and as tools for further investigations on the possible involvement of serotype-specific surface antigens in the virulence of these bacteria.

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