Use of Monoclonal Antibodies That Recognize p60 for Identification of *Listeria monocytogenes*

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*Listeria monocytogenes* causes major food-borne outbreaks of disease worldwide. Specific identification of this microorganism is of utmost importance to public health and industry. *Listeria* species are known to secrete a 60-kDa protein collectively termed p60, which is encoded by the *iap* (invasion-associated protein) gene and secreted in large quantities into the growth media. p60 is a highly immunogenic murein hydrolase that is essential for cell division. Due to these properties, p60 is an ideal diagnostic target for the development of immunological detection systems for *L. monocytogenes*. We report here two independent lines of monoclonal antibody (MAbs): p6007, which specifically recognizes *L. monocytogenes* p60, and p6017, which reacts with a wide range of *Listeria* p60 proteins. By combining these antibodies with a polyclonal antibody, we developed efficient sandwich enzyme-linked immunosorbent assay (ELISA) systems which can specifically identify *L. monocytogenes* or generally detect *Listeria* species. Since an excess amount of the peptide corresponding to PepA or PepD did not interfere with the ELISA, and direct ELISAs were unable to detect both peptides, we concluded that the epitope presumed to be recognized by p6007 or p6017 could be distinguished from PepA and PepD as described by Bubert et al. (Appl. Environ. Microbiol. 60:3120–3127, 1997). To our best knowledge, this is the first example of an immunological identification system that uses p60-recognizing MAbs.

The genus *Listeria* is comprised of six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. All *Listeria* species are widely found in nature as well as in many food industry-related applications (1). Among these species, *L. monocytogenes* is known to infect humans, causing sepsis, meningitis, or encephalitis (13). Due to the preferential infectivity of *Listeria* in infants, pregnant women, and immunocompromised patients and an associated high fatality rate, this organism can be classified as an important opportunistic infectious agent which is becoming an emerging problem in public hygiene (11, 12).

In order to prevent *L. monocytogenes* infection, prescreening of processed foods with reliable diagnostics is necessary. There have been a number of methods for detecting *L. monocytogenes* (5): conventional enrichment steps through the use of selective media or differential substrates followed by biochemical identification tests such as API Listeria, numerous types of PCR-based technology, including the recently described microarray-based assay (2, 15), and immunological assays (4). While the conventional methods are extremely time-consuming, the last two methods are relatively straightforward but require more sophisticated machines and discipline among the personnel conducting the laboratory testing. The PCR-based assays require extra steps such as electrophoresis or preparation of fluorescent probes, which can be tedious (8, 10). Nevertheless, because of their high levels of accuracy, these methods are gradually being implemented in diagnostic areas. Some immunological assays involving Western blotting and direct enzyme-linked immunosorbent assay (ELISA) that make use of a pair of polyclonal antibodies (PAb)s selectively recognizing *L. monocytogenes* p60 have been reported (4). The protein p60, which is encoded by the *iap* (invasion-associated protein)

| TABLE 1. Bacterial strains used |
|--------------------------------|
| Species | Strain(s)* |
|--------|-----------|
| *Listeria monocytogenes* | ATCC 19115, ATCC 19114, ATCC 7644, ATCC 15313, ATCC 19113, ATCC 19118, 12, 410, 530, SLCC R4, SLCC R1, SLCC M3 |
| *Listeria grayi* | ATCC 25401 |
| *Listeria welshimeri* | ATCC 35897 |
| *Listeria innocua* | ATCC 33090, NCTC 10528 |
| *Listeria ivanovii* | ATCC 19119 |
| *Listeria seeligeri* | ATCC 35967 |
| *Enterobacter aerogenes* | ATCC 13078 |
| *Escherichia coli* | ATCC 25922 |
| *Enterococcus faecalis* | ATCC 29212 |
| *Klebsiella pneumoniae* | ATCC 13882 |
| *Pseudomonas aeruginosa* | ATCC 27853 |
| *Staphylococcus aureus* | ATCC 25923 |
| *Staphylococcus epidermidis* | ATCC 29218 |
| *Streptococcus pyogenes* | ATCC 12022 |
| *Salmonella enterica serovar Typhi* | ATCC 19615 |
| *Vibrio cholerae* | KCTC 2126 |
| *Vibrio parahaemolyticus* | ATCC 17802 |

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* L. monocytogenes strains 3, 12, 410, and 539 were obtained from the National Veterinary Research and Quarantine Service. These strains were identified by established culture methods followed by PCR. Abbreviations: SLCC, Special Listeria Culture Collection; NCTC, National Collection of Type Cultures; KCTC, Korean Collection of Type Cultures.
gene, is secreted in large quantities in the culture supernatant of Listeria spp. p60 is murein hydrolase, which is essential for cell division, and is considered an important virulence factor (3, 9). It is a highly immunogenic protein (6). Due to these properties, p60 represents an ideal diagnostic target for development of immunological detection systems. However, the following factors may hinder the practical application of the aforementioned immunological assays to the diagnosis of L. monocytogenes: (i) due to possible low titers of the PAbs used, Western blot analysis must be performed with protein precipitation of a large volume of culture supernatant, and (ii) improper application of the sandwich ELISA format may limit the utility of the PAbs.

To cope with these pitfalls, we developed a panel of monoclonal antibodies (MAbs) recognizing L. monocytogenes p60 and immunological detection systems such as ELISA with the use of these MAbs. These tests enabled the effective detection of both laboratory and environmental strains of L. monocytogenes.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the origins of the Listeria and other bacterial strains used in this study. These strains were obtained from the American Type Culture Collection (Manassas, Va.), the Special Listeria Culture Collection (Wurzburg, Germany), the National Collection of Type Cultures (London, England), and the Korean Type Culture Collection (Daejon, Korea). The bacterial strains were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Listeria species were differentially identified by both API Listeria (Biomerieux, Marcy l’Etoile, France) and PCR by the use of specific primer sets whose sequences correspond to the p60 genes of Listeria species. Environmental strains of L. monocytogenes were isolated from cow feces by conventional culture...
methods and further identified by API Listeria. The cow feces were obtained from the National Veterinary Research and Quarantine Service.

Generation of the MAbs against *L. monocytogenes* p60. (i) Expression and purification of recombinant p60. The genes corresponding to the open reading frames of p60 were amplified from *L. monocytogenes* and *L. innocua* genomic DNA with a pair of primers. For *L. monocytogenes* p60, the forward primer sequence is 5'-GGG AAT TCC ATA TGA GCA CTG TAG TAG TCG AAG CT-3' and the reverse primer sequence is 5'-GCC GCT CGA GTA CGC GAC CGA AGC CAA C-3'. For *L. innocua*, the forward primer sequence is 5'-GGG AAT TCC ATA TGA GCA CAG TAG TAG TTG AAC T-3' and the reverse primer sequence is 5'-GCC GCT CGA GAG TTG GCT TCG GTC GCG TA-3'. PCRs were performed according to the standard protocol. The amplified fragments were digested with NdeI and XhoI and then cloned into pET21a (Novagen, Madison, Wis.). Recombinant p60 proteins were induced with a final concentration of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), purified as soluble forms through nitrilotriacetic acid-chelating agarose CL-6B (Peptron, Daejon, Korea), and then dialyzed against phosphate-buffered saline (PBS). Protein concentrations were determined with a commercial kit (Bio-Rad Laboratories, Hercules, Calif.), and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified protein was used to immunize rabbits (New Zealand White, female, 2 months old) along with complete Freund adjuvant followed by multiple boosting for PAb production.

(ii) Hybridoma production. Six-week-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, Mass.). The mice were repeatedly immunized with 20 μg of purified *L. monocytogenes* p60 plus complete Freund adjuvant. Prior to fusion, polysera were taken to test their reactivity with the p60 protein. Splenocytes were isolated and fused to the mouse myeloma cell line Sp2/0. The positive pools of hybridoma cells reacting with p60 were screened by direct ELISA. Single-cell clones were isolated by dilution.

(iii) Purification of antibodies. Ascites was obtained by injecting hybridoma cells into the peritoneal cavities of immunocompromised BALB/c mice. Polyclonal rabbit serum was obtained by bleeding from heart puncture. Antibodies were purified protein G columns. Immunoglobulin fractions were eluted and dialyzed against PBS. Protein concentrations were determined as described above.
FIG. 3. Comparison of binding characteristics between p6007 and p6017. (A) Different concentrations of recombinant p60 of *L. monocytogenes* and *L. innocua* were subjected to Western blot analysis using p6007. The intensity of each band was determined by an image analyzer and plotted in a semilog scale. Standard deviations (shown by error bars) were calculated from the results of three independent experiments. (B) Different concentrations of recombinant p60 of *L. monocytogenes* and *L. innocua* were subjected to Western blot analysis using p6017. The intensity of each band was determined as described for panel A.

**Western blotting.** Each *Listeria* species was grown in 5 ml of brain heart infusion broth to a turbidity of a McFarland’s nephelometer standard of >1.5. Ten microliters of culture broth was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, protein blots were incubated with p60 ascites or polyclonal rabbit serum diluted 1:2,000, at which dilution the signal-to-background ratio was maximal. Target protein bands were detected by chemiluminescence (Pierce Biotechnology, Rockford, Ill.).

**ELISA.** (i) Direct ELISA. Cell-free culture supernatants from *Listeria* spp. or other unrelated gram-positive or -negative bacterial strains were boiled for 10 min and used for the direct ELISA. Each well of a microtiter plate was coated with 100 µl of the boiled supernatant at 37°C for 2.5 h and then washed off. The plate was treated with a blocking buffer (PBS containing 1% bovine serum albumin and 0.05% Tween 20 [PBST]) for 1 h at room temperature. Diluted ascites or polyclonal serum was added to the plate, incubated at 37°C for 1 h, and washed three times with PBST. For colorimetric reactions, horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson Lab, West Grove, Pa.) diluted 1:1,000 in PBS and 2.2’-azino-bis(2-ethylbenzothiazoline)-6-sulfonic acid (Pierce) were used as substrates. The optical density (OD) was measured at 405 nm with an ELISA reader.

(ii) Sandwich ELISA. For the sandwich ELISA, each well was coated with 0.1 µg of p6007 or p6017 at 37°C for 2.5 h and blocked with PBST. One hundred microliters of the boiled supernatant was applied to each well; incubated at 37°C for 1 h, and washed three times with PBST. The secondary antibody was used at a concentration of 2 µg/ml at 37°C for 1 h. The experimental steps that followed were identical to those for the direct ELISA.

**RESULTS**

Generation of p6007 and p6017, two lines of MAb that recognize p60 of *L. monocytogenes* and a broad range of p60s of *Listeria* species, respectively. A panel of MAbs were generated and differentially screened for reactivity to recombinant p60 of *L. monocytogenes* or *L. innocua* by direct ELISA or Western blotting. Two MAbs, p6007 and p6017, were selected because of their unique properties, in that the former selectively recognized p60 of *L. monocytogenes*, whereas the latter recognized recombinant p60 from *L. monocytogenes* or *L. innocua*. To determine if these MAbs recognized the natural p60 proteins existing in the culture supernatants, we screened a panel of standard *Listeria* strains by sandwich ELISA using p6007 or p6017 as the capture antibody and a PAb recognizing *L. monocytogenes* p60 as the secondary antibody. To test the quality of the PAb used, we performed a series of direct ELISAs and Western blot analyses using the supernatants of the *Listeria* strains listed in Table 1. As shown in Fig. 1A, the PAb was able to recognize the p60 proteins from all strains tested except *L. grayi*, suggesting that the epitopes recognized by the PAb are largely conserved in various *Listeria* species and that the PAb can be used as the secondary antibody in sandwich ELISA. When paired with p6007, the sandwich ELISA selectively detected the p60 proteins from standard *L. monocytogenes* strains (Fig. 1B). Likewise, the p60 proteins from *L. monocytogenes* strains were unequivocally detected by Western blot analysis using p6007. On the other hand, as shown in Fig. 1C, the sandwich ELISA employing p6017 resulted in detection of the p60 proteins not only from all the *L. monocytogenes* strains but also from other *Listeria* species, including *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. However, Western blotting detected only the p60 proteins from *L. innocua*, *L. ivanovii*, and all *L. monocytogenes* strains among those tested, indicating that p6017 preferentially recognizes its epitope in the ELISA setting. These data suggest that p6007, p6017, or either one in combination with the PAb can be a useful detection tool for *L. monocytogenes* or other *Listeria* spp. To extend the usage of these MAbs to the environmental *L. monocytogenes* strains, 33 environmental strains originating in feces were subjected to identification by ELISA. The PAb clearly recognized the p60 proteins from all culture supernatants in Western blot analyses. Likewise, direct ELISA with this PAb produced significant levels of p60 from these culture supernatants (Fig. 2A). p6007 and p6017 produced similar patterns on West-
ern blots (Fig. 2B and C). More importantly, sandwich ELISA unequivocally identified these environmental strains as L. monocytogenes (Fig. 2B and C). These data once again confirm the diagnostic potential of both p6007 and p6017. To explore how much affinity or difference exists between p6007 and p6017, a set of binding kinetics assays was conducted by Western blotting followed by image analysis. As shown in Fig. 3A, while p6007 readily detected small amounts of recombinant p60 from L. monocytogenes, with 5 ng clearly seen, this antibody reacted slowly to recombinant p60 from L. innocua such that 50 ng of this p60 was barely detected even after prolonged exposure. A 100-fold difference in the degree of binding of p6007 to the corresponding p60 protein was observed when 50 ng was present, whereas the difference was only 10-fold with 1 μg. On the other hand, p6017 detected both p60 proteins to a similar extent. Taken together, these data demonstrate that p6007 is able to distinguish p60 proteins from L. monocytogenes and other Listeria spp. at typical concentrations in culture supernatants.

Recognition of the unique epitope by p6007 or p6017 distinguished from PepA or PepD. PepA and PepD are known to be unique epitopes recognized by a PAb isolated from rabbits immunized with p60 from L. monocytogenes culture supernatant (4). To determine whether p6007 or p6017 recognized these epitopes through direct ELISA and competitive sandwich ELISA, the reactions were performed in the presence of increasing concentrations of these peptides. As shown in Fig. 4A, our PAb recognized PepA but not PepD, suggesting that PepD does not contribute to recognition of p60 in sandwich ELISA. Neither MAb recognized PepA or PepD in direct ELISA when a concentration of 500 ng of peptide per well was used. The control peptide did not interfere either. These data suggest that p6007 and p6017 do not recognize PepA or PepD. Likewise, a competitive ELISA such as one using p6007 and PepA (Fig. 4B) or p6007 and p6017 (Fig. 4C) was not affected by these peptides when used in excess. These data suggest two possibilities: (i) neither PepA nor PepD is the epitope of p6007 or p6017, and (ii) even if the PAb clearly recognizes PepA in direct ELISA, the accessibility of p60 by the PAb in the sandwich ELISA format using p6007 with PAb might be unfavorable, possibly due to a conformational change in p60 induced by its binding to p6007.
DISCUSSION

Due to the physiological and genetic resemblance between Listeria species, specific detection of L. monocytogenes has been difficult. Recently, a real-time PCR-based detection assay called BAX and immunological assays such as VIDAS II or ELISA using PAbs have been implemented for detecting L. monocytogenes (7, 14). However, one pitfall of the use of these methods for detection is the cost of equipment. Since the commercially available ELISA system called Transia Plate makes use of a combination of PAbs, its sensitivity may be a limitation. For instance, the sandwich ELISA reported by Bu-bert et al. utilized a significant level of capture and detector antibodies, 20 μg/ml, and produced relatively weaker ODs than direct ELISA did (4). Moreover, from the standpoint of a manufacturer producing this ELISA system, the repeated production of enough PAbs may be cumbersome. Therefore, the MAAb-based sandwich ELISA solves these shortcomings. However, it was vital to discover whether the generation of L. monocytogenes p60-specific MAbs was possible before this study began. In the end, we generated a panel of MAbs against recombinant p60 and were able to retrieve two outstanding lines of MAbs, p6007 and p6017, in which the former specifically recognized L. monocytogenes p60 and the latter recognized a wide range of p60 proteins in Listeria species. We also produced a PAb against recombinant p60 from rabbits, which recognized the p60 proteins derived from a vast number of Listeria species. Given all of our experimental data from direct ELISA and competitive ELISAs in the presence of peptides corresponding to PepA or PepD, the epitopes of either of these MAbs are unlikely to be these peptides. Interestingly, although the PAb recognized PepA, it lost accessibility to PepA when combined with p6007. To our best knowledge, p6007 and p6017 are the first MAbs recognizing L. monocytogenes p60. We believe that these MAbs may demonstrate versatility for differential detection of L. monocytogenes.

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