Virulence of Culture Filtrate from Heat-Injured and Repaired *Listeria* Strains: Assay on Bovine Mammary Epithelial (MAC-T) Cells

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**ABSTRACT**

The cytotoxic effect of culture filtrates from healthy, heat-stressed, and repaired *Listeria monocytogenes* and *L. innocua* on the bovine mammary epithelial cell line MAC-T was examined. Culture filtrates were collected from *Listeria* spp. following treatments which included: (i) 18 h of growth of *Listeria* at 37°C; (ii) sublethal heat treatment at 56°C for 50 minutes; (iii) repair of the injured cells at 37°C for 7 h; (iv) growth of repaired bacterial cells at 37°C for 36 h; and (v) heat injury at 56°C for 50 min of the cell population obtained after the initial repair and growth. Strains chosen for study included two genetic mutants of *L. monocytogenes*: a hemolysin-negative mutant, CNL 85/162 (Hly−) and a hemolysin-positive revertant, CNL 85/163 (Hly+). Culture filtrates obtained from Hly+ bacteria did not prevent adherence of the mammary epithelial cells and slightly stimulated their growth. In contrast, culture filtrates from Hly− bacteria grown for 18 h significantly reduced the ability of MAC-T cells to adhere to the cell culture dishes, prevented the growth of those cells that were attached to the dishes, and caused cell death. Supernatants from Hly− and Hly+ following injury and during repair had no lethal effect on MAC-T cells. The effects of culture medium obtained after growth of the repaired *Listeria* cells on MAC-T cells were similar to those recorded for medium from the first 18 h growth for both strains, indicating that cells regain virulence potential once they have repaired and reinitiated growth. Culture filtrates obtained from *L. monocytogenes* Scott A showed results similar to those of Hly+, decreasing adherence and growth of MAC-T cells, while *L. innocua* culture filtrate had no adverse effect. The results of these experiments suggest that when injured, *L. monocytogenes* does not demonstrate adverse effects towards MAC-T cells. Once repair is completed and the *L. innocua* are growing, activity towards MAC-T cells is restored.

Key words: *Listeria*, MAC-T cells, virulence, heat injury, repair

*Listeria monocytogenes* is a facultative intracellular gram-positive bacterium which is pathogenic for both human and animals. Although *L. monocytogenes* has been isolated from a wide variety of fresh and processed foods, current methods underestimate its true incidence in foods because of the use of highly selective media which allow injured bacteria to escape detection. Injuries are followed by sublethal exposure of foodborne pathogens such as *L. monocytogenes* to heat, freezing, acids, or chemical sanitizers which are involved in the major methods of processing and preservation of foods. The question which arises is whether or not bacteria such as *Listeria* spp. are virulent in the injured state. Given favorable conditions, injured *L. monocytogenes* have the ability to repair and grow in foods, as was demonstrated by Meyer and Donnelly (24) for heat-injured *L. monocytogenes* in pasteurized whole and 2% (fat) milk stored at 4°C. Repair of sublethal injury in bacteria is often accompanied by restoration of pathogenicity, which could have serious public health implications.

All known virulent strains of *L. monocytogenes* produce hemolysin (listeriolysin), an extracellular protein belonging to the group of sulfhydryl-activated cytolysins. The role of listeriolysin as a major virulence determinant has been elucidated through a number of recent classic studies (10, 11, 12, 16, 18, 26). Therefore, listeriolysin has been implicated as a major virulence factor in listeric infections. Listeriolysin is not the only recognized virulence factor of *L. monocytogenes*. Kuhn and Goebel (19) identified another extracellular protein, p60, possibly involved in the attachment and invasion processes. In addition, mutants defective in the synthesis of phospholipase C can reportedly become avirulent (16). The production of superoxide dismutase and catalase by *Listeria* species has been associated with their ability to survive intracellularly by resisting oxidizing agents produced by phagocytes (3, 28).

The virulence of *Listeria* spp. has been determined frequently by oral, intravenous, or intraperitoneal inoculation of mice (2, 14, 15, 21). In these experiments, lethality in mice was the criterion used to determine the virulence of *Listeria*. The use of continuous cell lines is an alternative method for establishing the pathogenicity of *L. monocytogenes*. Bacteria-eukaryotic cell interactions such as adher-
ence, invasion, or killing of cell lines have been determined (8, 9, 20, 23, 25).

In this study, we determined whether bacterial culture filtrates from healthy, sublethally heat-stressed, and repaired \textit{Listeria} cells interfered with the adherence and/or growth of a bovine mammary epithelial cell line (MAC-T). An assay was developed using two strains of \textit{L. monocytogenes} known to have differences in virulence characteristics due to hemolysin production. The assay was validated using \textit{L. monocytogenes} Scott A, a clinical human isolate, and \textit{L. innocua} CWD 350, which was recovered from the environment of a food-processing plant. The objective of this study was to investigate the effects of heat injury on the virulence of \textit{L. monocytogenes} towards MAC-T cells.

**MATERIALS AND METHODS**

**Bacterial strains**

Two strains of \textit{L. monocytogenes} were received from Dr. J. L. Gaillard: CNL 85/162, a hemolysin negative (Hly-) strain obtained by transposon mutagenesis and CNL 85/163, a hemolysin-positive (Hly+) revertant strain obtained by spontaneous loss of the Hly-transposon (8). In addition, we used \textit{L. monocytogenes} Scott A, a human isolate received from Dr. V. K. Bunning (U.S. Food and Drug Administration, Laurel, MD) and \textit{L. innocua} CWD 350, an isolate obtained from a dairy plant environment. Stock cultures were stored frozen at \(-70^\circ\text{C}\) in Trypticase soy broth (BBL-Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 6% yeast extract (Difco Laboratories, Detroit, MI) and 10% glycerol (Difco).

**Growth medium**

\textit{Listeria} cells were grown, heat injured, and repaired in DL medium, composed of 75% Dulbecco's modified Eagle medium (DMEM) (Sigma Chemical Company, St. Louis, MO), and 25% listeria repair broth (LRB) without selective agents (5).

**Bacterial injury and repair**

\textit{Listeria} species were grown at \(37^\circ\text{C}\) for 18 h (primary growth) in 200 ml of DL medium. Cell populations following growth were enumerated on tryptose phosphate agar (TPA) (Difco). Bacterial cultures were centrifuged in a Sorvall RC-5B centrifuge (DuPont Coming, NY) and maintained at 37°C in a humidified (RH 100%), 5% CO\(_2\) incubator. Culture medium consisted of DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, Inc., Gaithersburg, MD) and 10% glycerol (Difco).

The supernatant was again set aside for further use. The bacterial cells were plated on TPA and TPAN in order to verify complete repair. The cells were then centrifuged as described above and the supernatant was used to inoculate 200 ml of DL medium which was then incubated at \(37^\circ\text{C}\) for 36 h (secondary growth). Repaired bacterial cells were plated on TPA and TPAN in order to verify complete repair. The cells were then centrifuged as described above and the supernatant was kept for further experiments. At the end of the 36-h period, \textit{L. monocytogenes} Hly\(^-\) and Hly\(^+\) were centrifuged and subjected to heat treatment (secondary injury) as previously described. All experiments were performed in duplicate.

**Cell-culture procedure**

Established bovine mammary epithelial cell lines (MAC-T) were maintained and cultured as described by Huynh et al. (17). Cells were routinely cultured in tissue-culture dishes (Corning Inc., Corning, NY) and maintained at \(37^\circ\text{C}\) in a humidified (RH 100%), 5% CO\(_2\) incubator. Culture medium consisted of DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY), 100 IU of penicillin (Sigma) per ml and 100 µg of streptomycin (Sigma) per ml. Antibiotics were added in order to prevent bacterial contamination. The medium was changed three times per week during regular maintenance of the cells. Medium was supplemented with 5% FBS and was changed every 24 h for experimental cultures.

**Cell-proliferation assay**

Approximately \(2 \times 10^4\) to \(5 \times 10^4\) MAC-T cells were plated onto each well of a 24-well culture dish in the presence of culture medium containing supernatant from the listeria primary and secondary growth, primary and secondary injury, and repair experiments. Prior to addition to the wells, these supernatants were filtered using 0.20-µm-pore-size acrodisc filters (Gelman Sciences, Ann Arbor, MI) in order to remove any bacteria present. Each treatment was tested in triplicate wells. For each experiment, the medium was made of one part DMEM and one part supernatant. A control was used in which supernatant was replaced by freshly made DL medium. The culture medium was supplemented with 5% FBS. Any remaining \textit{Listeria} cells were eliminated by the addition of 100 IU of penicillin per ml and 100 µg of streptomycin per ml.

MAC-T cells were grown for up to 3 days at \(37^\circ\text{C}\) in a humidified (100%) 5% CO\(_2\) incubator. The first 24 h of growth constituted the lag phase, and it was during this phase that the ability of MAC-T cells to adhere to culture dishes was assessed. Subsequent growth of MAC-T cells was determined by the counts obtained at 48 and 72 h from 3 control wells and 3 wells with each of the supernatants tested.

To recover the MAC-T cells from culture dishes for enumeration, the cells were incubated at \(37^\circ\text{C}\) with 0.05% trypsin (Sigma) containing 0.02% EDTA (Sigma) in calcium- and magnesium-free Hanks balanced salt solution (Sigma). Incubation was continued until MAC-T cells were released from the culture dishes. Viability of the MAC-T cells was determined by the trypan blue exclusion test in which 0.2% trypan blue (Sigma) was added to the cells, coloring only dead cells. Viable cells were counted using a hemocytometer. The average of two replicate counts per well was recorded.

**Statistical analysis**

An analysis of variance of our results was performed using a statistical analysis software package (SPSS-Version 4.1). The Duncan's multiple range test at a significance level of .05 was used to compare the effects of each \textit{Listeria} strain tested versus the control, by treatment. **RESULTS**

**Growth medium**

In order to assess the toxic effects of the \textit{Listeria} strains towards MAC-T cells, it was necessary to define cultural
conditions which would permit simultaneous cultivation of both *Listeria* and MAC-T cells. DMEM medium, although ideal for MAC-T cells, did not allow satisfactory growth of *L. monocytogenes*. In addition, LRB, a nonselective bacterial growth medium, was toxic to MAC-T cells. However, further work led to the development of DL medium, composed of 75% DMEM and 25% LRB. DL proved to be suitable for growth of both *Listeria* and MAC-T cells.

**Percent injury and repair**

Following sublethal heat treatments of 50 min at 56°C, we obtained injury of the majority of the *Listeria* population (99.9%), and this was consistent for all *Listeria* strains tested. Injury results were recorded for the primary as well as the secondary injury. Heat-stressed bacteria were able to completely repair in DL medium within 7 h of incubation at 37°C. As indicated for Hly+ and Hly− (Fig. 1), repair was deemed complete when counts obtained on both TPA and TPAN plates were similar.

**Cell proliferation assays**

*a. Assay development.* We examined the effects of filtrates from five distinct physiological phases of *Listeria* strains (Table 1). Virulence was defined as the inability of MAC-T cells to grow and form monolayers, whereas lack of virulence was defined as the absence of adverse effects on MAC-T cells (Fig. 2).

Filtrate from the primary growth of Hly+ interfered with growth and monolayer development of MAC-T cells (Fig. 3). Counts at 48 h (2.2 × 10⁴ cells/ml) and 72 h (1.5 × 10⁴ cells/ml) indicated that there was a significant reduction (*P* < .05) in the number of cells. By contrast, culture filtrate from primary growth of the *L. monocytogenes* Hly− showed no adverse effects. In fact, MAC-T cells grew significantly

**TABLE 1. Effects on MAC-T cells of culture filtrates from *Listeria* strains after different treatments**

<table>
<thead>
<tr>
<th><em>Listeria</em> culture filtrate after:</th>
<th>Hly+</th>
<th>Scott A</th>
<th>Hly−</th>
<th><em>L. innocua</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary growth</td>
<td>killed</td>
<td>growth stimulated</td>
<td>killed</td>
<td>growth stimulated</td>
</tr>
<tr>
<td>Primary injury</td>
<td>growth stimulated</td>
<td>growth stimulated</td>
<td>growth stimulated</td>
<td>growth stimulated</td>
</tr>
<tr>
<td>Repair</td>
<td>growth stimulated</td>
<td>growth slowed</td>
<td>growth stimulated</td>
<td>no effect</td>
</tr>
<tr>
<td>Secondary growth</td>
<td>killed</td>
<td>killed</td>
<td>growth stimulated</td>
<td>growth stimulated</td>
</tr>
<tr>
<td>Secondary injury</td>
<td>growth stimulated</td>
<td>ND*</td>
<td>growth stimulated</td>
<td>no effect</td>
</tr>
</tbody>
</table>

*ND, not determined.*
more \((P < .05)\) than control cells grown in sterile DL medium.

Results from primary injury indicate that no adverse morphological effects were observed on MAC-T cells when filtrates of both Hly\(^+\) and Hly\(^-\) were added to the assay wells (Fig. 4). Surprisingly, MAC-T cells grew equally well and significantly more than the control in the culture filtrates from both sublethally heat-treated \(L.\) monocytogenes Hly\(^+\) and Hly\(^-\) strains \((1.0 \times 10^5\) and \(9.5 \times 10^4\) cells/ml respectively at 72 h). Therefore, DL medium from injured listeria contained elements which had a stimulatory effect on the growth of MAC-T cells.

Fig. 5 illustrates the effects of filtered supernatant collected following repair of heat-injured \(L.\) monocytogenes Hly\(^+\) and Hly\(^-\) on MAC-T cells. Supernatant obtained from both strains showed identical results in that there was no effect in terms of adherence of MAC-T cells to the culture dishes, but once again, in comparison to the control, there was a significant increase of MAC-T cell growth over 72 h \((1.3\) and \(1.2 \times 10^5\) cells/ml).

Subsequent experiments were conducted in an attempt to determine if following the first sublethal heat treatment \(L.\) monocytogenes Hly\(^+\) and Hly\(^-\) would acquire heat resistance and subsequently affect MAC-T cells. Filtered supernatants obtained following secondary growth and secondary injury of both strains of \(L.\) monocytogenes had the same effects on MAC-T cells as filtrates from primary growth and primary injury, respectively (data not shown). In contrast to the Hly\(^-\) strain, new growth of the Hly\(^+\) strain prevented adherence and growth of MAC-T cells, and both strains, when thermally stressed, stimulated growth of MAC-T cells.

b. Strain comparison. Culture filtrates obtained from primary and secondary growth of \(L.\) monocytogenes Scott A significantly reduced \((P < .05)\) adherence of MAC-T cells...
to culture dishes and their subsequent growth, measured at 72 h (Fig. 6). The number of MAC-T cells decreased from 9.0 × 10^5/ml (control) to 1.0 × 10^5/ml (Scott A). In addition, filtrates collected following heat-injury treatment significantly increased (P < .05) growth of MAC-T cells over a 72-h period (1.1 × 10^6 cells/ml). However, unlike Hly^+, filtrate from repaired L. monocytogenes Scott A significantly inhibited growth of MAC-T cells.

Supernatants obtained after primary and secondary growth as well as after heat injury and repair of L. innocua CWD 350 had no significant adverse effect on MAC-T cells (P > .05) as compared to the control (Fig. 7). This indicates that avirulent Listeria strains show no lethal effects and confirms the utility of MAC-T cells for evaluating the virulence potential of Listeria strains.

**DISCUSSION**

The major finding emerging from this study is that the MAC-T cell line proved useful for assessing the virulence potential of Listeria strains as a function of their physiological state: healthy, injured, or repaired. Heat-injured L. monocytogenes were able to regain their ability to kill MAC-T cells once they fully repaired and reintegrated growth. Looking at the two genetically modified strains of L. monocytogenes CNL 85/163 (Hly^+) and CNL 85/162 (Hly^-), we found that filtrates from growing cell cultures affected proliferation of MAC-T cells differently from culture filtrates from injured or repaired listeria cells. Furthermore, there was a significant difference in the behavior of both strains towards MAC-T cells, confirming a major role for hemolysin in the pathogenicity of L. monocytogenes. DL medium filtered after primary and secondary growth of Hly^- favored the growth of mammary epithelial cells as shown by improved adherence and subsequent proliferation. This finding suggests that by-products from metabolism had a stimulating effect on MAC-T cells. In contrast, culture filtrates obtained following primary and secondary growth of the strain Hly^+ were very unfavorable for the mammary epithelial cells: in fact most MAC-T cells died (80% reduction).

Based on known differences between the strains Hly^+ and Hly^- used in this study, production of hemolysin appears to be detrimental to MAC-T cells. Several authors have noted that loss of hemolysin production in L. monocytogenes is often associated with loss of virulence. Gaillard et al. (10), who engineered the two strains with which we worked, reported that the hemolysin-negative L. monocytogenes strain was completely avirulent in mice. After spontaneous loss of the transposon, virulence was restored in this strain which reverted to hemolysin producing (10). In a similar study by Kathariou et al. (18), loss of virulence, as tested in a mouse model, paralleled inactivation of hemolysin production in a virulent strain of L. monocytogenes. As expected, the revertant strain regained virulence (18).

L. monocytogenes Scott A and L. innocua CWD 350 were used in order to determine if the results from the assay developed with the strains Hly^+ and Hly^- could be related to behavior of wild-type Listeria spp. As our experiments indicated, we were successful in our trials. L. monocytogenes Scott A behaved generally in the same manner as the Hly^+ strain. Filtered supernatants obtained from primary and secondary growth significantly reduced adherence of MAC-T cells to culture dishes and their subsequent growth. However, there was a major difference between the strains Scott A and Hly^- in the effects of culture filtrates obtained following the 7-h repair period of the bacterial cells. While Hly^- stimulated the growth of MAC-T cells, Scott A inhibited the growth of MAC-T cells but did not kill them. This difference might be linked to the ability of L. monocytogenes Scott A to cause human clinical illness.

L. innocua CWD 350 treatments had an outcome different from L. monocytogenes Scott A. These results resemble those obtained with treatments of L. monocytogenes Hly^-, in that culture filtrates collected from L. innocua had no adverse effect on adherence or growth of MAC-T cells. Supernatants obtained after primary and secondary growth as well as after repair had no significant effect on MAC-T cells.

These results correlate with those obtained by Farber and Speirs (8), in which culture filtrates from hemolysin-producing L. monocytogenes and L. ivanovii were reportedly cytotoxic for eight different cell lines. The authors also found that culture filtrates from L. innocua, a nonhemolytic Listeria species, had no cytotoxic effect on the same cell lines.

The ability of heat-injured bacterial pathogens to regain virulence was demonstrated in a study by Collins-Thompson et al. (6), who subjected Staphylococcus aureus strain S6 to a sublethal heat stress of 52°C for 15 min and assessed the production of enterotoxin B after repair of the heat-injured cells. Their results demonstrate complete recovery of S. aureus following heat treatment, including the ability to synthesize enterotoxin B (6). McCarthy (22) studied the pathogenicity of nonstressed, heat-stressed, and resuscitated L. monocytogenes 1A towards immunocompromised mice and reported that the same doses (10^3) of nonstressed or
resuscitated cells were sufficient to cause infection in mice, indicating that the latter had regained pathogenicity.

It should be noted that filtered supernatants obtained following heat injury of all Listeria strains tested, with the exception of L. monocytogenes Scott A, had a positive effect on the growth of MAC-T cells. Although proteins and amino acids from heat-injured L. monocytogenes were not detected in previous examinations of culture supernatants (4), an explanation for this phenomenon might be that intracellular material released by bacteria which died during the heat treatment was favorable for the growth of MAC-T cells. In her study with immunocompromised mice, McCarthy (22) demonstrated that a higher dose of heat-stressed cells was necessary for pathogenicity, suggesting that virulence factors were lost due to injury.

Although additional work needs to be done to assess the direct effects of heat-treated and repaired Listeria cells on MAC-T cells, several conclusions can be drawn from this study. The first is that any extracellular virulence factors produced by the hemolytic revertant of L. monocytogenes Hly⁺ and by the clinical isolate Scott A were not exhibited when cells were heat injured, or even during the process of repair. Most importantly, those extracellular virulence factors were once again produced when repaired Listeria cells were allowed to grow. Therefore, to ensure the absence of injured Listeria cells which may repair and regain pathogenic potential in foods, steps should be taken to detect both healthy and injured L. monocytogenes in food products. This could be accomplished by the addition of a repair step prior to the selective enrichment period included in the isolation procedures of Listeria spp. from foods endorsed by the FDA and the USDA-FSIS.

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