Examination of Cytopathic Effect and Apoptosis in *Listeria monocytogenes*-Infected Hybridoma B-Lymphocyte (Ped-2E9) Line *In Vitro*

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Abstract In our previous studies, we reported that hybridoma B-lymphocytes can be used to determine the virulence of *Listeria* species in an *in vitro* cytotoxicity assay. Here, we examined the cytopathic effect, i.e., membrane damage and the nature of cell death induced by *Listeria monocytogenes* on murine hybridoma B-lymphocytes (Ped-2E9). Membrane damage was assessed by microscopic analyses and by measuring the release of intracellular alkaline phosphatase (AP) and lactate dehydrogenase (LDH). Cell death was determined by DNA fragmentation analyses using agarose gel electrophoresis. Infection by listeriolysin O (LLO)-producing *L. monocytogenes* strains induced substantial amounts of AP and LDH release from Ped-2E9 hybridoma B-cells, suggesting severe membrane damage in these cells, while an LLO-negative *L. monocytogenes* mutant strain had no effect. An LLO-producing recombinant *L. innocua* (*prfA$^+$hly$^+$*) strain also induced high AP and LDH release and cytopathic changes in Ped-2E9 cells. Light or scanning electron microscopic examination revealed *L. monocytogenes* mediated membrane destabilization, pore formation, intense cytoplasmic granulation, bleb formation, and lysis of Ped-2E9 cells. LLO-producing *L. monocytogenes* and *L. innocua* (*prfA$^+$hly$^+$*) also induced ladder-like DNA fragmentation in Ped-2E9 cells. Collectively, these results suggest that *L. monocytogenes*, specifically LLO-producing strains, can induce a severe cytopathic effect leading to apoptosis in hybridoma B-lymphocytes (Ped-2E9).

Key words: *Listeria monocytogenes*, hybridoma B-lymphocyte, alkaline phosphatase, lactate dehydrogenase, DNA fragmentation, apoptosis, microscopy

Listeria monocytogenes is a deadly foodborne bacterial pathogen and causes listeriosis in immunocompromised individuals with symptoms such as meningitis, encephalitis, septicemia, and liver abscess [33]. Occasionally, this organism can cause gastroenteritis in healthy adults, and abortion and stillbirth in pregnant women [32, 33]. The pathogenic mechanism and cytotoxicity effect of this facultative intracellular bacterial pathogen is often studied by using various mammalian cells [10]. The cell lines may include epithelial [13, 15-17], dendritic [21], macrophage [1, 12], and those of B-lymphocyte origin such as NS1, Ped-2E9, EM-7G1, and RI.37 [4-6].

Among the genus *Listeria,* only *L. monocytogenes* is known to be pathogenic for humans and possesses numerous virulent factors for intracellular survival and cell-to-cell spread [29]. Among the virulent factors, listeriolysin (LLO), encoded by the *hly* gene and regulated by *prfA* (positive regulatory gene), was shown to be an important factor responsible for phagosomal membrane pore formation in both professional or nonprofessional phagocytic cells [2, 8, 24, 37]. As a result, *L. monocytogenes* cells can escape into the cytoplasm and induce actin-based bacterial motility. In addition, LLO was also reported to induce apoptosis in hepatocytes [31], dendritic cells [21], and T-lymphocytes [27].

In our previous studies, we reported that *L. monocytogenes* induced a cytotoxic effect on hybridoma B-lymphocytes, and this cytotoxic property was utilized to develop an *in vitro* rapid cytotoxicity assay for *Listeria* species [4, 6]. The major objectives in this study were to investigate in detail the nature of cytopathic effects, i.e., the membrane damage and cell death (apoptosis) induced by *L. monocytogenes* on Ped-2E9 hybridoma B-cells. Membrane damage and the nature of cell death were assessed by measuring the release of endogenous alkaline phosphatase (AP) and lactate dehydrogenase (LDH), using light and scanning electron microscopy, and a DNA fragmentation assay. In addition, we also examined the role of LLO in a cytopathic effect and apoptosis. Understanding the detailed mechanism of cytotpathic effects will help to design an efficient and rapid *in vitro* cytotoxicity model for *L. monocytogenes.*

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MATERIALS AND METHODS

Listeria Cultures

L. monocytogenes strains Scott A (serotype 4b), SLCC 5764 (serotype 1/2a), hemolysin (listeriolysin) negative M12 (derived from SLCC 5764) [24], and hemolysin-producing recombinant *L. innocua* strain pERL3-503 (*prfA$^+$hly$^+$*) [14] were maintained and grown in appropriate antibiotic media. For experimental purposes, cultures were subcultured twice in brain heart infusion (BHI) broth at 37°C, washed twice in 20 mM phosphate buffered saline, pH 7.0 (PBS), and resuspended in PBS to obtain cell populations of 1×10^9 CFU/ml. The cultures were also tested for hemolysin production on 5% sheep blood agar plates.

Hybridoma B-Lymphocytes (Ped-2E9) and Inoculation with *Listeria* Species

Murine hybridoma Ped-2E9 cells were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (DMEM-FBS, Sigma) as previously reported [4]. Ped-2E9 cells were grown in 75-cm^2 flasks, harvested, and resuspended in media with or without serum to obtain cell populations of 2 to 2.5×10^6/ml. Ped-2E9 cells were infected with bacteria at a multiplicity of infection (MOI) of 100 bacteria per host cell and incubated at 37°C for 6 h in all experiments unless otherwise indicated. The MOI and exposure time were previously determined to be optimum for observing *Listeria*-induced hybridoma B-cell cytotoxicity [4].

Alkaline Phosphatase (AP) and Lactate Dehydrogenase (LDH) Assays

Ped-2E9 cells were incubated with 0.1 ml test organisms (1×10^9 CFU/ml), and the supernatants were collected and assayed for AP according to the method described previously [6, 19]. Briefly, samples were centrifuged (300 ×g, 5 min) and 0.1 ml of cell supernatants was distributed in several wells of a 96-well microtiter plate. A 0.1 ml aliquot of an AP substrate solution (0.1 M Tris, 0.1 M NaCl, 5 mM MgCl$_2$, pH 9.5) containing *p*-nitrophenyl phosphate (PNPP, 1 mg/ml, Sigma) was added to each well. The plates were read (A$_{405/595}$) after 3 to 5 min of incubation using a Bio-Rad (Hercules, U.S.A.) plate reader. The LDH activities of the supernatants were analyzed by using a cytotoxicity assay kit from Boehringer Mannheim (Indianapolis, U.S.A.).

Light and Scanning Electron Microscopy

The Ped-2E9 cells (1×10^7/ml) were inoculated with *Listeria* sp. and incubated at 37°C. For light microscopic analysis, the Ped-2E9 cells were stained with trypan blue after 4 h of incubation and examined. For scanning electron microscopy (SEM), the cell suspensions, after 2 h of incubation, were centrifuged (300 ×g, 10 min) and washed three times to remove any free bacteria. The cells were fixed with 2.5% (v/v) glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4), post-fixed with 2% (v/v) OsO$_4$, rinsed with water, and lyophilized using liquid nitrogen. The cells were finally sputter-coated with gold-palladium and examined under a JSM 6400 scanning electron microscope [11].

Analysis of DNA Fragmentation

After exposure to *Listeria* sp., the Ped-2E9 cells were washed once in PBS and lysed with 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.5% Triton-X for 30 min. The samples were treated with proteinase K (0.5 mg/ml) at 50°C for 2 h and RNase (0.25 mg/ml) at 42°C for 2 h. DNA was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, and analyzed using agarose (1.5%) gel electrophoresis [31]. Additional Ped-2E9 cells were also incubated at 44°C for 30 min to induce DNA fragmentation and apoptosis, and used as a positive control [26].

RESULTS AND DISCUSSION

Membrane Damage Analysis by AP and LDH Release Assay

AP is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein and is abundant in activated B-lymphocytes [19, 35]. It is a large protein of about M_r 100 to 190 kDa [6, 30]. Conversely, LDH is a low molecular weight cytoplasmic enzyme (M_r 35 kDa) that can exist in tetrameric form and is also present in lymphoid cells [7]. The release of such large molecular weight AP and low molecular weight LDH after exposure to *Listeria* sp. was employed to evaluate the severity of membrane damage and pore formations in Ped-2E9 hybridoma B-cells *in vitro*. We observed that *L. monocytogenes* WT strains (Scott A and SLCC 5764) induced large amounts of AP (56 to 91%) and LDH (83 to 100%) to be released from hybridoma Ped-2E9 cells, suggesting severe membrane damage in these cells. In contrast, a nonpathogenic *L. innocua* strain did not cause any discernible enzyme release (Table 1). To specifically evaluate the role of LLO in membrane damage and cytotoxicity, we tested a LLO deficient *L. monocytogenes* M12 (Δ*hly*) strain [24]. The data presented in Table 1 shows that the M12 strain did not produce any AP or LDH release, indicating that LLO was responsible for Ped-2E9 membrane damage. The significant role of LLO in membrane damage was further confirmed by the use of an LLO-producing recombinant *L. innocua* strain pERL-3 harboring *prfA* and *hly* genes [14], which showed 71% AP and 86% LDH, similar to values from *L. monocytogenes* WT strains (Table 1). Collectively, these results suggest that LLO is responsible for membrane damage and pore formations in hybridoma Ped-2E9 cells

Table 1. Effect of *Listeria monocytogenes* and recombinant *L. innocua* strains on viability and alkaline phosphatase (AP) and lactate dehydrogenase (LDH) release from Ped-2E9 hybridoma B-cells[a].

Strains[b]	Genotype	Hemolytic activity[c]	Viable count×10^5/ml	AP[d] ($A_{405/595}$)	%	LDH[d] ($A_{490/655}$)	%
Control (PBS)			22.5±0.82	0.20±0	0	0.37±0.01	0
L. monocytogenes							
Scott A (4b)	WT	+	5.87±0.42	0.92±0	91	1.10±0.03	100
SLCC 5764 (1/2a)	WT	+	12.7±0.77	0.64±0.02	56	0.92±0	83
M12	*Δhly*	−	21.9±0.32	0.20±0	0	0.30±0	0
L. innocua							
NCTC11288	WT	−	21.6±0.77	0.20±0	0	0.39±0	3
pERL3-503	*prfA+hly+*	+	0.33±0.03	0.76±0.06	71	0.94±0.03	86

[a]All values are mean±the standard error of mean (SEM) of two separate experiments and analyzed in duplicate (n=8).

[b]*L. monocytogenes* M12 is a transposon (Tn*916*)-induced mutant strain derived from SLCC 5764 and *L. innocua* pERL3-503 is a recombinant strain derived from the NCTC 11288 strain.

[c]Hemolytic activity was determined on sheep blood (5%) agar plate.

[d]Triton-X (0.5%)-treated Ped-2E9 cells had AP and LDH absorbances of 0.99±0.09 and 1.03±0.04, respectively. Percent AP or LDH was calculated as $(Abs_{Exp} - Abs_{Control})/(Abs_{Triton-X} - Abs_{Control}) \times 100$.

resulting in increased AP and LDH release. A similar observation was made by Sibelius *et al.* [34], who reported that LLO was also responsible for severe cell membrane damage in human umbilical vein endothelial cells.

In B-cells, the majority of cellular AP exists as a GPI-anchored protein in the membrane [19], therefore, only a low level of AP was expected to be released in the supernatant following *Listeria* sp. infection. However, we found substantial amounts of AP being released from the Ped-2E9 cells (Table 1). It could be postulated that the release of AP from cells could be partly facilitated by the cleavage of GPI by listerial PI-PLC (phosphatidylinositol-specific phospholipase C), which possibly could gain access to the interior of cells through the pores generated by LLO. However, listerial PI-PLC has a very low activity against GPI [18], thus the action of PI-PLC alone may not be sufficient to cause high levels of AP release. Alternatively, the most plausible explanation for high levels of AP release could be due to the effect of LLO, which definitely induces severe membrane damage and disintegration (severe membrane damage was also confirmed by microscopic examinations; Figs. 1 and 2), and allows the release of an intact GPI-anchored AP complex from the membrane into the surrounding environment, resulting in high AP activity. For LDH activity, it has been reported that LDH molecules are freely present in the cytoplasm of most of the mammalian cells [7, 30] that are released, due to membrane pore formations.

LLO has been reported to be a membrane pore-forming toxin, which binds to cholesterol and causes the lysis of red blood cells [20]. LLO is also known to form pores in the phagosomal membrane after the entry of *L. monocytogenes* into host cells [2, 29]. In hybridoma Ped-2E9 cells, LLO possibly causes transmembrane pore formations and there allows the release of intracellular molecules. There are several other toxins, including leukotoxins from *Pasteurella*

hemolytica [11] and *Escherichia coli* [3, 22], and a-toxin from *Staphylococcus aureus* [23], that are also reported to cause the lysis of target cells by the formation of transmembrane pores. The pore formation leads to increased membrane permeability, loss of essential ions and molecules, and cell death.

Cell Membrane Damage and Cell Death Analysis by Light and Scanning Electron Microscopy

A light microscopic examination of hybridoma Ped-2E9 cells revealed that *L. monocytogenes* Scott A induced multiple bleb formation, cytoplasmic granulation, membrane disintegration, and a partial to complete lysis of the cells (Figs. 1B, 1C, and 1D). LLO-positive recombinant *L. innocua* *prfA+hly+* also caused similar cytopathic changes (Figs. 1G and 1H). Control Ped-2E9 cells or cells treated with *L. innocua* WT remained viable without any visible cytopathic changes (Figs. 1A and 1F). Ped-2E9 cells treated with heat (44°C for 30 min) showed similar bleb formations as in the *L. monocytogenes*-treated cells (Fig. 1E). Scanning electron micrographs also revealed dramatic cytopathic changes in exposed hybridoma Ped-2E9 cells (Fig. 2). Cells infected by *L. monocytogenes* Scott A exhibited numerous pores in the membrane and, in some cases, the pores were very large (Figs. 2C and 2D). Honeycomb-like structures were also visible in the cell membrane and, in some cases, cells appeared to be shrunken. Infection by LLO-producing recombinant *L. innocua* *prfA+hly+* also induced severe cytopathic changes (Figs. 2E and 2F) similar to *L. monocytogenes* Scott A. Control Ped-2E9 cells appeared as smooth and spherical (Fig. 2A), as did *L. innocua*-treated cells which appeared as normal with a slightly rough surface structure without any visible pores or membrane damage (Fig. 2B). Again, these microscopic observations confirmed that LLO is the primary hemolysin responsible for membrane damage, pore formation, and cell lysis in Ped-2E9 cells.
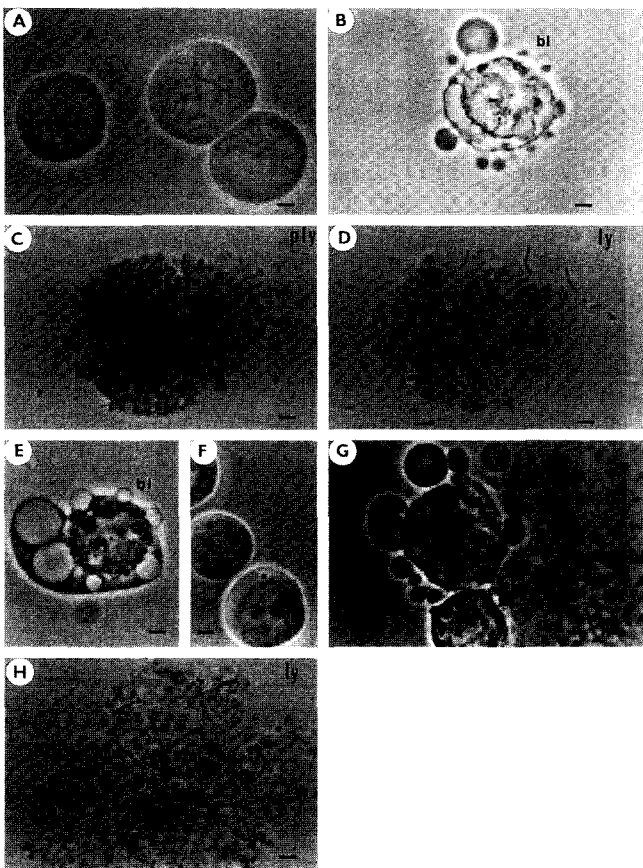
Fig. 1. Light microscopic photographs of Ped-2E9 hybridoma B-cells showing cytopathic changes induced by *Listeria* sp. after 4 h of exposure.

Photographic panels are: (A) control Ped-2E9 cells; (B, C, and D) cells treated with *L. monocytogenes* Scott A; (E) heat treatment (44°C, 30 min), (F) *L. innocua* NCTC 11288; and (G and H) *L. innocua* (*prfA⁺hly⁺*). Both *L. monocytogenes* and recombinant *L. innocua* (*prfA⁺hly⁺*) strains showed identical cytopathic effects as to the bleb formation (bl), membrane destabilization, cytoplasmic granulation, and partial (ply) to complete lysis (ly) of the cells. Heat treatment also induced a bleb formation similar to the *L. monocytogenes* and *L. innocua* (*prfA⁺hly⁺*) strains. In contrast, *L. innocua* WT-treated and control Ped-2E9 cells remained spherical and smooth without any apparent morphological changes (bar, 1 μm).

DNA Fragmentation Analysis and Apoptosis

We investigated the nature of cell death in *Listeria*-infected Ped-2E9 cells. It has been reported that many microorganisms or their toxins cause apoptosis in a variety of cells with characteristic membrane blebbing, chromatin condensation and margination, and DNA fragmentation [9]. In this study, we observed that in hybridoma Ped-2E9 cells, hemolysin producing both *L. monocytogenes* and *L. innocua prfA⁺hly⁺* strains induced ladder-like DNA fragmentation, while controls (PBS and *L. innocua*) had no effect (Fig. 3). Ped-2E9 cells, when treated with heat (44°C for 30 min), induced a DNA fragmentation pattern consistent with apoptosis (Fig. 3). Several previous studies have indicated that *L. monocytogenes* or LLO induces apoptosis in hepatocytes [31], dendritic cells [21], and lymphocytes
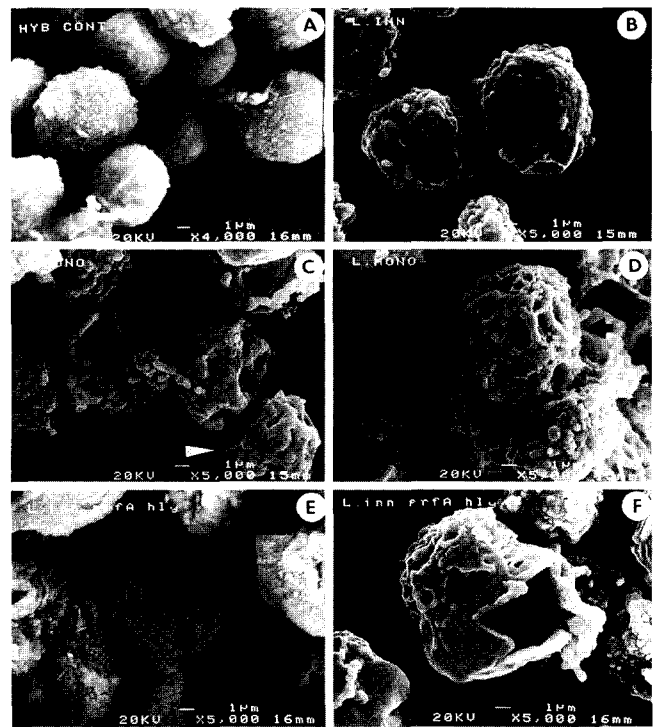

Fig. 2. Scanning electron photomicrograph of Ped-2E9 hybridoma B-cells after exposure to *Listeria* sp. for 2 h.

Photographic panels are: (A) control; (B) *L. innocua* NCTC 11288; (C and D) *L. monocytogenes* Scott A; and (E and F) *L. innocua* (*prfA⁺hly⁺*). *L. monocytogenes* Scott A and *L. innocua* (*prfA⁺hly⁺*) produced multiple pores. In some cases, cells appeared shrunken (white arrow), produced honeycomb-like structures (dark solid arrow), and lost their cellular integrity, whereas the control cells remained healthy (bar, 1 μm).

[27], yet does not elicit apoptosis in bone marrow-derived macrophages [1]. Apoptosis in T-lymphocytes has been reported to be induced by several bacterial toxins, including staphylococcal α-toxin [23], *E. coli* hemolysin [22], and *P. haemolytica* leukotoxin [11, 36]. B-cells were also reported to manifest spontaneous apoptosis under various growth conditions such as prolonged cultivation [35] or the absence of growth factor IL-6 [25]. However, there are no reports on bacteria or their toxin-mediated apoptosis in B-cells. In this study, we documented for the first time that LLO-producing *L. monocytogenes* or recombinant *L. innocua* (*prfA⁺hly⁺*) elicited blebbing, pore formation, the destabilization of the cytoplasmic membrane, cytoplasmic granulation, and nuclear fragmentation in Ped-2E9 hybridoma B-lymphocytes, leading to apoptosis. Additionally, the signs of apoptosis induced by *L. monocytogenes* were identical to the apoptosis characters (cytological and DNA fragmentation) induced by heat treatment [26].

In conclusion, this study demonstrated that the *L. monocytogenes*-mediated cytotoxic effect in hybridoma B-cells *in vitro*, indeed led to apoptosis in these cells. Furthermore, LLO was the primary virulent factor that was
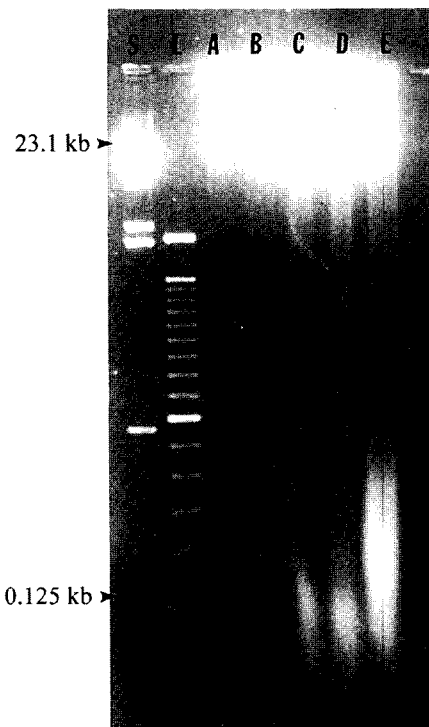
Fig. 3. Agarose gel electrophoresis of DNA fragmentation from Ped-2E9 cells after treatment with *Listeria* sp. for 6 h.

Lanes are: (S) *Hind*III-digested λ DNA standard (23.1–0.125 kb); (L) DNA ladder (100 bp); (A) Ped-2E9 cells alone; (B) *L. innocua* NCTC 11288; (C) *L. innocua prfA⁺hly⁺*; (D) *L. monocytogenes* Scott A; and (E) heat treatment at 44°C for 30 min. *L. monocytogenes, L. innocua prfA⁺hly⁺*, and heat treatment elicited DNA fragmentation while controls (cells alone or *L. innocua*) did not.

responsible for apoptotic cell death in Ped-2E9 cells. However, it remains to be investigated whether *L. monocytogenes* is capable of inducing a similar cytotoxic effect leading to apoptosis in B-cells *in vivo*, since this facultatively intracellular bacterium is known to provoke T-cell mediated cellular immunity in a host.

Acknowledgments

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