Listeria monocytogenes listeriolysin O and phosphatidylinositol-specific phospholipase C affect adherence to epithelial cells

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Abstract: Listeria monocytogenes, a foodborn intracellular animal and human pathogen, produces several exotoxins contributing to virulence. Among these are listeriolysin O (LLO), a pore-forming cholesterol-dependent hemolysin, and a phosphatidylinositol-specific phospholipase C (PI-PLC). LLO is known to play an important role in the escape of bacteria from the primary phagocytic vacuole of macrophages, and PI-PLC supports this process. Evidence is accumulating that LLO and PI-PLC are multifunctional virulence factors with many important roles in the host–parasite interaction other than phagosomal membrane disruption. LLO and PI-PLC may induce a number of host cell responses by modulating signal transduction of infected cells via intracellular Ca²⁺ levels and the metabolism of phospholipids. This would result in the activation of host phospholipase C and protein kinase C. In the present study, using Bacillus subtilis strains expressing LLO, PI-PLC, and simultaneously LLO and PI-PLC, we show that LLO and PI-PLC enhance bacterial binding to epithelial cells Int407, with LLO being necessary and PI-PLC playing an accessory role. The results of this work suggest that these two listerial proteins act on epithelial cells prior to internalization.

Key words: Listeria monocytogenes, listeriolysin O, phosphatidylinositol-specific phospholipase C, Bacillus subtilis, adherence.

Résumé : Listeria monocytogenes, un pathogène intracellulaire animal et humain de source alimentaire, produit plusieurs exotoxines contribuant à la virulence. On retrouve parmi celles-ci la listériolysine O (LLO), une hémolysine formant des pores dépendants du cholestérol, et une phospholipase C spécifique au phosphatidylinositol (PI-PLC). LLO est reconnu comme un important acteur dans la fuite de la bactérie de la vacuole phagocytaire primaire des macrophages et la PI-PLC soutient ce processus. De plus en plus de preuves indiquent que la LLO et la PI-PLC sont des facteurs de virulence multifonctionnels jouant plusieurs rôles importants dans l’interaction hôte–parasite autres que la déchirure de la membrane phagosomale. La LLO et la PI-PLC pourraient induire une variété de réponses de la cellule-hôte en modulant la transduction des signaux des cellules infectées à travers les niveaux de Ca²⁺ intracellulaire et le métabolisme des phospholipides. Ceci entraînerait l’activation de la phospholipase C de l’hôte et la protéine kinase C. Dans l’étude présente, en utilisant des souches de Bacillus subtilis exprimant LLO, PI-PLC, et LLO et PI-PLC simultanément, nous avons démontré que la LLO et la PI-PLC favorisent l’attachement des bactéries aux cellules épithéliales Int407, LLO étant nécessaire et PI-PLC jouant un rôle accessoire. Les résultats de ces travaux indiquent que ces deux protéines listériales agissent sur les cellules épithéliales avant l’internalisation.

Mots clés : Listeria monocytogenes, listériolysine O, phospholipase C spécifique au phosphatidylinositol, Bacillus subtilis, adhérence.

Introduction

Listeria monocytogenes is a facultative intracellular bacterial pathogen that is widely distributed in the environment and that can cause severe food-borne infections such as meningitis, encephalitis, and septicemia in humans (McLauchlin 1997). Virulent strains of L. monocytogenes are able to invade, survive, and multiply within both nonprofessional phagocytic host cells, such as hepatocytes and epithelial cells, and professional phagocytes, such as macrophages (Gaillard et al.1987; Dramsi et al. 1995; Drevets et al. 1995). Entry of L. monocytogenes into epithelial cells by a process similar to phagocytosis occurs through the interaction of internalins InlA and InlB proteins with their cellular receptors (Gaillard et al. 1991; Mengaud et al. 1996; Shen et al. 2000). Following internalization, the bacteria escape from the phagosomal vacuole, proliferate in the cytosol, and exploit host actin-based motility to spread to adjacent cells by means of filopodium-like projections. At this stage of infection, the bacteria are transiently confined in double-membrane vacuoles, from which they escape to repeat the cycle (Tilney and...
Portnoy 1989; Dabiri et al. 1990; Mounier et al. 1990; Theriot et al. 1992). The most important factor involved in the escape of bacteria from phagocytic vacuoles is the thiol-activated pore-forming hemolysin listeriolsyn O (LLO) (Gaillard et al. 1987; Mengaud et al. 1988; Bielecki et al. 1990). Another virulence factor, a phosphatidylinositol-specific phospholipase C (PI-PLC), aids LLO in escape from the primary vacuole (Camilli et al. 1993). Recent findings indicate that LLO and PI-PLC may have an impact on modulating signal transduction pathways prior to the entry of L. monocytogenes into host cells. Mutants lacking LLO and PI-PLC displayed significant defects in initiation of host-cell signaling in host human umbilical vein endothelial cells (HUVEC), indicating that one of these proteins alone was not enough to induce maximal phosphoinositide metabolism (Sibelius et al. 1996a, 1996b). LLO allows access of the bacterial PI-PLC to host phosphatidylinositol (PI), thus leading to the formation of inositol-P (InsP) and diacylglycerol (DG) (Sibelius et al. 1996a, 1999; Goldfine et al. 2000).

The second messengers originated as a result of LLO and PI-PLC to mediate adhesion of bacteria to epithelial cells, and LLO and PI-PLC to mediate adhesion of bacteria to epithelial cells in the absence of other listerial factors contributing to pathogenesis.

The results presented herein indicate that LLO and PI-PLC influence bacterial binding to epithelial cells, and LLO is indispensable for this process, whereas PI-PLC plays a less important role.

**Materials and methods**

**Bacterial strains and plasmids**

Wild-type L. monocytogenes 10403S, serotype 1/2a, was used for these studies. Bacillus subtilis strains DP-B1066 (carrying pAG58-ble-1 vector alone) and DP-B1340 (expressing isopropyl-β-D-thiogalactopyranoside- (IPTG)- inducible PI-PLC) have been described elsewhere (Camilli et al. 1991), as have parental B. subtilis strain ZB307 (Zuber and Losick 1987) and plasmid pAG58-ble-1 (Youngman et al. 1989). The strains, as well as plasmid pAG58-ble-1, were kindly provided by Dr. D. Portnoy (University of California, Berkeley). Escherichia coli MC1061 was used in cloning experiments. Plasmids constructed for this study are described in detail below. Bacillus subtilis recombinant strains expressing IPTG-inducible LLO and IPTG-inducible LLO and PI-PLC were obtained in this study. The properties of B. subtilis strains, as well as plasmids used in this study, are summarized in Table 1.

**Growth conditions, media, and reagents**

*Bacillus subtilis* was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) at 37 °C. For the experiments, B. subtilis strains were grown overnight with shaking at 37 °C. The following morning, the culture was diluted 1:20 into fresh medium. These cultures were grown at 37 °C with aeration to *A*<sub>600</sub> 0.55. In the case of *B. subtilis*, chloramphenicol (10 μg·mL<sup>-1</sup>), phleomycin (0.8 μg·mL<sup>-1</sup>), and IPTG (1 mmol·L<sup>-1</sup> final concentration) were added to broth when required. *Escherichia coli* was cultured in Luria–Bertani medium (Difco) at 37 °C with ampicillin (100 μg·mL<sup>-1</sup>). Restriction enzymes, Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from Roche. Taq DNA polymerase was obtained from Qiagen. All reagents were used according to the manufacturer’s instructions.

**Plasmid construction**

The L. monocytogenes 10403S *hly* gene was cloned into pAG58-ble-1. Sense primer (5′-EcoRV site underline) 5′-GGCGGATATCGAGAGGGCTGCAAAACGGT-3′ and antisense primer (5′-SalI-KpnI site underline) 5′-GCTGTCGGCAGGTACCTTTGCTGTGGTTAAGCGGTG-3′ were used to amplify, using polymerase chain reactions (PCRs), a 1.7-kb DNA fragment representing the *hly* gene from the chromosomal DNA of *L. monocytogenes* previously digested with endonucleases *SalI* and *KpnI*. PCR was performed in a Thermal Cycler (MJ Research, Inc.) for 35 cycles of 94 °C for 2 min, 49 °C for 1 min in the first cycle and 69 °C for 1 min in following cycles, and 72 °C for 2 min. The amplified LLO-encoding DNA fragment was digested with *EcoRV* and *SalI* and, after purification with a PCR Purification Kit (Qiagen), ligated to plasmid pAG58-ble-1 digested with HindIII (the ends were made blunt with Klenow fragment of DNA polymerase I after digestion) and *SalI*, forming plasmid pAG58-ble-1-*hly*. The plcA gene was cloned into pAG58-ble-1-*hly* digested with endonucleases *SalI* and *KpnI*. Briefly, sense primer (5′-KpnI site underline) 5′-GGCGGATATCGAGAGGGCTGCAAAACGGT-3′ and antisense primer (5′-SalI-BglII site underline) 5′-GGCTGTCGGCAGGTACCTTTGCTGTGGTTAAGCGGTG-3′ were used to amplify a PI-PLC encoding 1.0-kb DNA fragment. PCR was performed for 35 cycles of 94 °C for 2 min, 55 °C for 1 min, and 72 °C for 1.5 min. The amplified DNA fragment was digested with *KpnI* and *SalI* and, after purification with the PCR Purification Kit (Qiagen), was ligated to plasmid pAG58-ble-1-*hly*. All plasmids constructed in this study were confirmed to be proper by restriction analysis.

**Transformation of E. coli and B. subtilis**

*Escherichia coli* transformation was performed as described by Sambrook et al. (1989), and *B. subtilis* transformation was performed by the competent cell method (Anagnostopoulos and Spizizen 1961).

**Western blot analysis of LLO and PI-PLC secretion by recombinant B. subtilis strains**

The overnight cultures of *B. subtilis* strains were diluted 1:20 into fresh BHI broth supplemented with 1 mmol·L<sup>-1</sup> IPTG and grown at 37 °C with aeration to *A*<sub>600</sub> 0.55. Culture supernatants were collected by centrifugation and secreted.
proteins were precipitated with trichloroacetic acid (at a final concentration of 10%) for 1 h at 4 °C. The precipitate was pelleted, washed with acetone, and resuspended in the final sample buffer. The samples were subjected to sodium dodecyl sulfate – 10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The positions of molecular mass standards (LMW, Pharmacia) were detected after the blot was stained with Ponceau S red (Sigma). The blot was developed with alkaline-phosphatase-conjugated goat anti-rabbit PI-PLC and anti-LLO sera, respectively. The blot was detected after the blot was stained with Ponceau S red (Sigma). The blot was developed with alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin.

**Hemolytic activity produced by *B. subtilis* strains**

Hemolytic activity was determined by the level of hemoglobin released from 1% sheep red blood cells (SRBC). Briefly, a sample (1 mL) of culture, grown as described above, was subjected to centrifugation, and 20 µL of the supernatant fluid was incubated with 1% SRBC in a total volume of 1% Triton X-100. Results are the average of three independent experiments, each carried out in triplicate.

**Tissue culture and growth medium**

Human embryonic intestinal epithelial cell line Int407 was kindly provided by E. Jagusztyn-Krynicka (Warsaw University, Warsaw, Poland). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum decomplemented for 30 min at 56 °C, 2 mmol·L⁻¹ L-glutamine, penicillin (100 U·mL⁻¹), and streptomycin (10 µg·mL⁻¹) in 100-mm Petri dishes and were fed three times a week with 10 mL of medium. Cells were propagated by splitting 1:5 into fresh media followed by treatment with trypsin. The cells were kept in a humidified incubator at 37 °C and supplied with a mixture of 5% CO₂ – 95% air. The growth medium was changed to antibiotic-free medium the day before infection.

**Adhesion assay and FACS analysis**

To examine the interaction of recombinant *B. subtilis* strains with epithelial cells, fluorescence-activated flow cytometry (FACS) was performed as previously described (de Boer et al. 1996). Briefly, monolayers of cell line Int407 were washed twice by adding 10-mL portions of PBS to culture plates. Cells were detached from the plates by vigorous agitation. Cells were counted with a haemocytometer and adjusted to a concentration of 1 × 10⁶ viable cells per 200 µL with HEPES (2-N-(2-hydroxyethyl-piperazine-N’-(2-ethanesulfonic acid) buffered Ringer medium, containing 1 mmol·L⁻¹ Ca²⁺ (pH 7.4), in a round-bottom polypropylene tube. The cell suspensions were allowed to equilibrate at 37 °C in a 6% CO₂ atmosphere for 1 h before bacteria were added. *Bacillus subtilis* (1 mL) was washed twice with PBS (pH 7.4) before infection (MOI 10) of Int407 cells (supplemented with 1 mmol·L⁻¹ IPTG). After 30 min of incubation with gentle swirling at 37 °C, bacteria that had not adhered were removed from the assay system by centrifugal washes (10 min at 700g), once in 15% saccharose solution (in PBS pH 7.4) and twice in PBS. To recognize cell-associated *B. subtilis*, cells were resuspended in PBS containing 0.5% bovine serum albumin (Sigma) and incubated for 30 min with polyclonal rabbit anti-*B. subtilis* ZB307 antibodies (1:1000; Kucharczyk T.E.). After two washes with PBS, the cells were incubated with allophycocyanin (APC)-conjugated goat anti-rabbit antibodies for 30 min (1:300; Merck). Subsequently, after two further washes, the cells were fixed in 500 µL paraformaldehyde solution (1% in PBS). The level of fluorescence of infected cells was measured with a FACSscalibur flow cytometer (Becton Dickinson); results confirmed in three independent experiments were analyzed using CELLQuest software (Becton Dickinson).
Expression of LLO and PI-PLC by *B. subtilis* strains

The gene encoding LLO was cloned downstream from the IPTG-inducible promoter $p_{spac}$ present in plasmid pAG58-ble-1 as previously described for LLO (Bielecki et al. 1990), except that the cloned sequence excludes promoter and terminator region of *hly* gene. The transcriptional fusion of *hly* and *plcA* genes was constructed by cloning gene encoding PI-PLC downstream from the LLO gene present in plasmid pAG58-ble-1- *hly*.

After transformation to parental *B. subtilis* strain ZB307, Southern blot analysis confirmed the integration of each plasmid into the *B. subtilis* chromosome, which occurs as a result of the presence of the pBR322-derived sequence in both pAG58-ble-1 and the Spβ prophage in *B. subtilis* ZB307, providing homology for integrative recombination (Youngman et al. 1989; Zuber and Losick 1987). The resulting *B. subtilis* strains carrying *hly* and *hly* plus *plcA* secreted LLO and LLO plus PI-PLC, respectively, after the induction of expression with IPTG, as indicated by Western blot analysis (Figs. 1A and 1B). After carrying out hemolysis studies, we found that LLO and PI-PLC secreted by the *B. subtilis* recombinant strains cause membrane permeabilization of SRBC in an acidified (pH 5.6) environment (Fig. 2), but not in a neutral (pH 7.4) environment (data not shown). In an acidified environment, the *B. subtilis* strain expressing LLO and PI-PLC exhibited over twofold higher hemolytic activity compared with the strain expressing LLO alone. However, the role of PI-PLC in hemolysis was auxiliary to LLO activity, since the strain secreting PI-PLC and the strain secreting no listerial protein produced an identical, very low level of hemolytic activity that seems to correspond to unspecific hemolysis (Fig. 2). These results are in good agreement with the well-known properties of LLO and PI-PLC (Goldfine et al. 1995).

Binding of recombinant *B. subtilis* strains expressing LLO and simultaneously, LLO and PI-PLC, to Int407 cells is significantly increased compared with *B. subtilis* strain expressing PI-PLC alone

To verify the possible influence of LLO and PI-PLC on bacterial binding to epithelial cells in the absence of other listerial virulence factors, the binding of recombinant *B. subtilis* strains to Int407 cells was performed. Bacteria bound to epithelial cells were labeled using APC-conjugated antibodies and visualized by FACS analysis (Fig. 3). Expression of LLO caused a ~15%–20% increase in cell binding, and simultaneous expression of LLO and PI-PLC caused a ~50% increase, a 60% increase in cell binding relative to that of the *B. subtilis* strain expressing no listerial virulence factor. The intensity of fluorescence Int407 cells infected with the *B. subtilis* strain expressing only PI-PLC was not significantly different from the *B. subtilis* strain secreting no listerial protein (Fig. 3). To rule out the possibility that differences observed in fluorescence intensity were caused by differences in antibody binding among the tested strains, Western blot analysis using anti- *B. subtilis* antibodies was
performed, and no differences were detected in the supernatants or surface-binding proteins expressed by B. subtilis recombinant strains (data not shown). These results clearly show that LLO facilitates bacterial binding to epithelial cells and that PI-PLC cooperates with LLO in this process, though it alone brings about no changes.

Discussion

The objectives of this research were to investigate the role and potential cooperation between LLO and PI-PLC in the early stages of the infection process. It is well-known that PI-PLC supports LLO in many processes involved in pathogenesis, e.g., membrane permeabilization (Goldfine et al. 1995), the escape of L. monocytogenes from the primary vacuole (Bannam and Goldfine 1999; Camilli et al. 1993), and the induction of host cell intracellular signalling (Sibelius et al. 1996a, 1999; Wadsworth and Goldfine 1999, 2002). The present study has demonstrated that L. monocytogenes PI-PLC cooperates with LLO, since the highest level of hemolytic activity and increased adhesion were observed in the case of B. subtilis expressing LLO and PI-PLC; lower, albeit significant, levels in the case of B. subtilis expressing LLO; and no essential changes in the case of B. subtilis expressing PI-PLC alone. These findings suggest that LLO is necessary for these processes, whereas PI-PLC plays a minor role.

In the case of the macrophage model of infection with L. monocytogenes, it was demonstrated that LLO and PI-PLC delay adhesion and entry into macrophage-like cells J774 and subsequently facilitate escape from the primary vacuole, and that changes in cellular signaling occur before association with, and entry of, bacteria into these cells (Wadsworth and Goldfine 1999). The work presented in this paper demonstrates that LLO and PI-PLC influence bacterial association with epithelial cells. However, in contrast with changes caused by LLO and PI-PLC in macrophage-like cells J774 (Wadsworth and Goldfine 1999), we observed accelerated bacterial binding to epithelial cells by LLO and PI-PLC in the Int407 cell lines. These results suggest that the activity of LLO and PI-PLC may be different, depending on the type of eukaryotic cells lines being used. The uptake of L. monocytogenes by normally nonphagocytic epithelial cells is triggered, with the internalins playing the major role in this process (Gaillard et al. 1991, 1996). The use in these studies of a B. subtilis strain, i.e., bacteria not producing any internalins, enabled to observe the participation of LLO and PI-PLC in the binding of bacterial cells to epithelial cells. The observed ability of LLO and PI-PLC to accelerate adhesion to naturally nonphagocytic epithelial cells is beneficial for bacteria, and probably, in the case of L. monocytogenes, facilitates the achievement of optimal level of invasion and intracellular growth. Previous work indicated that LLO and PI-PLC can change host cell intracellular signalling extra-

Fig. 3. Determination of Bacillus subtilis recombinant strains binding to intestinal epithelial cell line Int407 by FACS. Histograms show the relative log fluorescence intensity of Int407 cells incubated with DP-B1340 B. subtilis strain expressing PI-PLC (red line), AK-B101 B. subtilis strain expressing LLO (green line), and AK-B105 B. subtilis strain expressing LLO and PI-PLC (blue line). The black line corresponds to fluorescence intensity caused by DP-B1066 B. subtilis strain with vector alone. Specific binding of bacteria was detected with polyclonal rabbit anti-B. subtilis ZB307 antibodies and APC-conjugated secondary antibodies.
cellularly by producing synergistic effects on the release of inositol phosphates and DAG (Sibelius et al. 1996a, 1999). It was shown that human umbilical endothelial cells, which did not internalize L. monocytogenes, produced products of phosphoinositide hydrolysis when incubated with listeriae that produced LLO alone or LLO plus PI-PLC (Sibelius et al. 1996a, 1996b). Wadsworth and Goldfine (1999) showed that LLO and PI-PLC are essential for initiating rapid increases in calcium levels in the J774 murine macrophage cell line and, more recently, demonstrated that both these molecules are required for the translocation of protein kinase C δ (PKC δ) and protein kinase β II (PKC II), and that LLO and PI-PLC modulate host signalling pathways before internalization of bacteria occurs, providing an optimal level of uptake and subsequently the escape of bacteria from the primary phagocytic vacuoles of J774 cells (Wadsworth and Goldfine 2002). On the other hand, Repp et al. (2002) demonstrated that LLO applied extracellularly causes long-lasting oscillations of the intracellular Ca2+ level in human embryonic kidney cells as a result of a pulsed influx of extracellular Ca2+ through pores that are formed by LLO in the plasma membrane. It is possible that the delivery system for PI-PLC proposed by Sibelius et al. (1996a), in which LLO enables PI-PLC translocation into the cytosol of infected cells and which is analogous to the type-III secretion system of Gram-negative bacteria (Madden et al. 2001), is responsible for the observed changes in adhesion caused by LLO and PI-PLC, since the expression of PI-PLC increases the adhesion of B. subtilis strains to epithelial cells only in the presence of LLO. The hypothesis that changes in the adhesion of bacteria to the surface of epithelial cells are mediated by LLO and PI-PLC secreted to the environment is additionally supported by the fact that the Western blot method revealed that only a small amount of PI-PLC, but not of LLO, remains bound to the bacterial cell surface (data not presented).

To summarize, the present work indicates that LLO and PI-PLC enhance bacterial binding to epithelial cells in the absence of well-known listerial factors involved in this phenomenon, and PI-PLC supports LLO in the above-mentioned process. These observations are indicative of the important role of LLO and PI-PLC in the early stages of infection of nonprofessional phagocytic cells by L. monocytogenes.

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References


