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Mammalian Cells Transfected with the Listeriolysin Gene Exhibit Enhanced Proliferation and Focus Formation

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Mouse 3T6 and 3T3 fibroblasts and rat epithelial L2 cells were transfected with recombinant plasmids containing the listeriolysin gene (*hly*) of *Listeria monocytogenes*. This bacterial gene (with and without the 5′ signal sequence) was cloned under the control of a murine metallothionein promoter, resulting in elevated transcription of both forms of the *hly* gene after induction with $ZnSO_4$. However, the gene product could be observed only when the listeriolysin gene lacking the 5′ signal sequence was used. Intact listeriolysin could not be detected in the cytoplasm or in the supernatant of the *hly*-transfected cells. 3T6 and L2 cells transfected with the intact *hly* gene exhibited significantly increased cell proliferation and increased formation of actin microfilaments upon induction of *hly* expression with $ZnSO_4$. Both cell types are not contact inhibited and formed large piles of spherical cells after transfection with *hly*. In contrast, contact-inhibited 3T3 cells transfected with the *hly* gene showed increased proliferation but no formation of such cell aggregates. When 3T6 fibroblasts were transfected with the *hly* gene without the 5′ signal sequence, inhibition of growth, lack of cell layer confluency, and altered (spherical) cell morphology were observed.

Listeriolysin, a bacterial toxin produced by all virulent *Listeria monocytogenes* strains, belongs to the group of sulfhydryl-activated cytolysins (1, 4, 42). These membrane-active proteins recognize cholesterol as a receptor and form pores of as yet undefined size in cholesterol-containing biological and artificial membranes (6, 7). Recent studies have shown that this toxin is an essential virulence factor of *L. monocytogenes* (16, 20, 25, 29), required for their release from the phagosome into the cytoplasm (8, 25, 39, 40). It is assumed that the pore-forming property of listeriolysin causes the disruption of the phagosomal membrane together with a phosphatidylinositol-specific phospholipase C (PlcA) produced by virulent *L. monocytogenes* strains (11, 12, 45). The gene encoding listeriolysin (*hly*) is coordinately regulated with several other clustered virulence genes by the positive transcription factor PrfA (13, 33). In addition, expression of listeriolysin is enhanced by elevated temperature and repressed by cellobiose and low pH (17, 32, 38), suggesting that environmental parameters also influence the expression of this toxin. Our own unpublished data indicate that preferential synthesis of listeriolysin occurs in the phagosomal compartment (22). Its probable requirement for cell-to-cell spread (disruption of the double membrane envelope formed after entry of the bacteria into the neighboring cell) suggests, however, that listeriolysin is also synthesized at later times in the intracellular infection cycle.

In order to study other possible cellular effects of this toxin without the interference by other listerial virulence factors, we transfected the listeriolysin gene, *hly*, into fibroblast and epithelial cell lines. Here, we show that mouse fibroblast 3T3 and 3T6 and rat epithelial L2 cell lines transfected with the intact listeriolysin gene exhibited enhanced cell proliferation. Furthermore, formation of cell aggregates was observed in the non-contact-inhibited cell lines 3T6 and L2 but not in the contact-inhibited 3T3 cell line upon induction of listeriolysin gene expression. In contrast, 3T6 cells expressing listeriolysin without the N-terminal signal sequence exhibited reduced proliferation and cell damage.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The listeriolysin gene and an ovalbumin gene were cloned into either pUC- or pMa/pMc5-8-based plasmids and then transferred into *Escherichia coli* HB101 for expression in bacteria. Expression of the listeriolysin and ovalbumin genes in mammalian cells was performed with the cDNA expression vector pBMG *neo*. The plasmids pUC18, pMa/pMc5-8, and pBMG *neo* have been previously described (24, 43, 52).

Construction of plasmids for the expression of listeriolysin from *L. monocytogenes* EGD Sv 1/2 a. Plasmid pLM47, provided by M. Leimeister-Wächter (31), was used as a source for the wild-type *hly* gene. For the expression of listeriolysin in mammalian cells, an *Xba*I site was generated by site-specific mutagenesis, using the plasmid system pMa5-8/pMc5-8 (43) and an oligonucleotide with the sequence 5′-GCATTATTAG GTTAAAAAATCTAGA-3′ 20 bp upstream of the initiation codon ATG of *hly*. A 1,670-bp fragment containing the complete *hly* gene was generated by digesting the mutagenesis vector pMc5-8 (pMc5-8 *hly*) with *Xba*I. The *Xba*I fragment was blunt end ligated between the intron and poly(A) signals of the rabbit β-globin gene of the expression vector pBMG *neo* (24, 37, 44), resulting in plasmid pBMG *neo hly*. Plasmid pBMG *neo* ΔS *hly* was generated by deleting the *hly* amino-terminal signal sequence by *Nco*I digestion.

The pUC derivatives were constructed by inserting the *Hin*dIII-*Bam*HI fragment carrying the complete neomycin and listeriolysin expression cassettes that also contained the metallothionein promoter into the multiple cloning site of pUC18.

Standard protocols were used in the preparation of plasmid DNA, cleavage, ligation, and transformation of competent bacteria (34).

Tissue cultures and growth medium. Cell lines used included the rat epithelial cell line L2 (ATCC CCL 149) and the mouse fibroblast cell lines 3T6 (ATCC CCL 96) and 3T3

(ATCC CCL (2). These were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. All medium components were supplied by GIBCO. The cell lines were grown at 37°C in a humid atmosphere containing 5% CO_2.

Cytotoxicity assay. A total of 10^4 3T6 fibroblasts or L2 epithelial cells per well (wells A to H) were seeded in 200 μl of Dulbecco's modified Eagle's medium in a 96-well plate and incubated for 12 h at 37°C. The protein concentrations from overnight cultures of *Listeria innocua* 6b, *L. monocytogenes* EGD Sv 1/2a, and *L. monocytogenes* NCTC 7973 were measured by the method of Bradford (9). Equal amounts (100 μg/ml) of protein from each culture in a final volume of 200 μl were added to well A. After the listerial supernatant and cell medium were mixed, a serial dilution was performed by transferring 200 μl from well A to well B, from well B to well C, and so on. After 6 h, the cytolytic titer was measured by washing the cells in each well twice with phosphate-buffered saline (PBS) and staining the remnant of adhering cells with trypan blue.

Transfection of 3T6, 3T3, and L2 cells. Transfection of mammalian cells by $CaPO_4$ precipitation was carried out as previously described (21). For the selection of stable transfectants, cells were trypsinized 48 h after transfection, and G418 (GIBCO) was added to the growth medium at a concentration of 400 μg/ml (3T6 and 3T3 fibroblasts) or 600 μg/ml (L2 cells). Experimental procedures were performed after the stably transfected cells were passaged five times.

Isolation of DNA from transfected cells. High-molecular-weight DNA was isolated as described by Wigler et al. (51). Hirt extracts (low-molecular-weight DNA) were prepared as described elsewhere (23).

PCR analysis. The M197 (5′-GCTGCTTTTGATGCTGCC GTAGACGG-3′) and M200 (5′-CTATATTTCGGATAAAG CGTGGTGCCCC-3′) PCR primers were synthesized by laboratory facilities. Template DNA was isolated as described above. PCR (41) was performed with an initial denaturation step of 3 min at 94°C, and then 30 cycles were run as follows: 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C. Reaction products were run on 1% agarose gels.

RNA isolation and Northern (RNA) blotting. Total RNA was isolated from transfected cells of individual 90-mm-diameter plates by the guanidine thiocyanate-cesium chloride method described by Chirgwin et al. (14).

A total of 10 μg of RNA per lane was run using denaturing agarose gels as described previously (34). After electrophoresis, RNA was transferred to Hybond-N membranes in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer as described previously (46). Membranes were prehybridized for 6 h and then hybridized for 12 h with the ^{32}P-labeled 1,670-bp *Xba*I fragment from pMc5-8 *hly*. Hybridization was performed in a solution of 6× SSC, 5× Denhardt's solution, 0.2% (wt/vol) sodium dodecyl sulfate (SDS), and 100 μg of sonicated carrier DNA per ml in sealed plastic bags at 65°C. Filters were then washed in 0.2× SSC–0.1% (wt/vol) SDS for 30 min at 45°C and exposed for autoradiography.

SDS-PAGE and immunoblots. Total eukaryotic cell proteins were prepared from 10^7 cells from overnight cultures. The cells were washed twice in PBS, harvested by being scraped into 1 ml of PBS, pelleted by centrifugation for 5 min at 4°C, lysed with 200 μl of lysis buffer (10 mM Tris [pH 7.8], 150 mM NaCl, 600 mM KCl, 0.5 mM $MgCl_2$, 2% Triton X-100), and sonicated for 10 s. These cell extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (30). Transfer of proteins to nitrocellulose and subsequent processing of the blots were performed as described by Towbin et al. (47). Antilisteriolysin rabbit antibody was used as the primary antibody at a dilution of 1:500 in immunoblots. Blots were developed by using horseradish peroxidase-conjugated anti-rabbit antisera (Dako), 0.015% hydrogen peroxide, and 4-chloro-naphthol as substrates.

Cell proliferation assay. For the cell proliferation assay, 10^5 cells in 3 ml of Dulbecco's modified Eagle's medium were cultured in 60-mm-diameter culture dishes for 24 h. After induction of the transfected cells with $ZnSO_4$ (90 μM) for 12 h, 100 μl of [^{3}H]thymidine (1.0 μCi/ml; Amersham) per ml of culture medium was added. Medium was removed after various periods, and the cells were washed with ice-cold 1× Hanks balanced salt solution. Next, the cells were incubated for 10 min at 20°C with 6 ml of 10% trichloroacetic acid and washed twice with 1× Hanks balanced salt solution. After the washes, the culture dishes were incubated for 30 min at 60°C with 1.5 ml of 0.3 N NaOH–1% SDS. Samples were cooled to room temperature, and thymidine incorporation was measured (18).

RESULTS

Construction of recombinant plasmids carrying intact or leaderless listeriolysin (*hly*) genes and their transfection into mouse fibroblast 3T6 and 3T3 and rat epithelial L2 cell lines. Insertion of the intact *hly* gene and its truncated derivative lacking the 5′ signal sequence was performed in the papillomavirus vector pBMG *neo* as shown in Fig. 1. Both constructs should allow the expression of the inserted *hly* genes under the control of the murine metallothionein promoter. In addition, the *hly* and *neo* genes and the metallothionein promoter were inserted in the vector pUC18 so that the *hly* gene was controlled by the metallothionein promoter (Fig. 1). The *hly* plasmids were transfected into 3T6, 3T3, and L2 cells, and the localization of the *hly* gene in each cell type was analyzed by PCR (Fig. 2). While positive signals were obtained in Hirt extracts of papillomavirus plasmid-transformed cells, *hly*-specific signals were observed only in the nuclear pellets of cells transfected with the pUC18 plasmid constructs (Fig. 2). The data thus demonstrate that the papillomavirus plasmids allow the episomal establishment of the *hly* genes in the transfected mammalian cells, whereas the pUC18 constructs integrate the *hly* genes into the genome of these cells.

Induced expression of the *hly* genes in the presence of $ZnSO_4$. Expression of the listeriolysin genes in transfected eukaryotic cells grown to the logarithmic or stationary phase was measured by examining the *hly*-specific transcripts and the expected gene products following induction by $ZnSO_4$. Although *hly*-specific mRNA was detected before induction of 3T6 cells transfected with pBMG *neo hly* (Fig. 3A, lane 3), the synthesis of this transcript was significantly induced upon treatment of the cells with 90 μM $ZnSO_4$ (Fig. 3A, lane 4). Cells transfected with the pUC18 derivatives synthesized detectable amounts of *hly*-specific mRNA only upon induction with $ZnSO_4$ (Fig. 3B, lane 4), suggesting that considerably more copies of the *hly* gene are present in the pBMG *neo hly*-transfected cells. While mRNAs of the intact and the 5′ leaderless *hly* genes were synthesized in similar amounts (data not shown), only the listeriolysin protein without the signal sequence was detected by Western blot (immunoblot) analysis. Moreover, the protein was detected solely in the cytosolic fraction of the transfected cells (Fig. 4) and not in the supernatant or the membrane fraction of these cells. We also tried to detect LLO expression in the *hly*-transfected cells by immunoprecipitation, but we failed to observe the intact

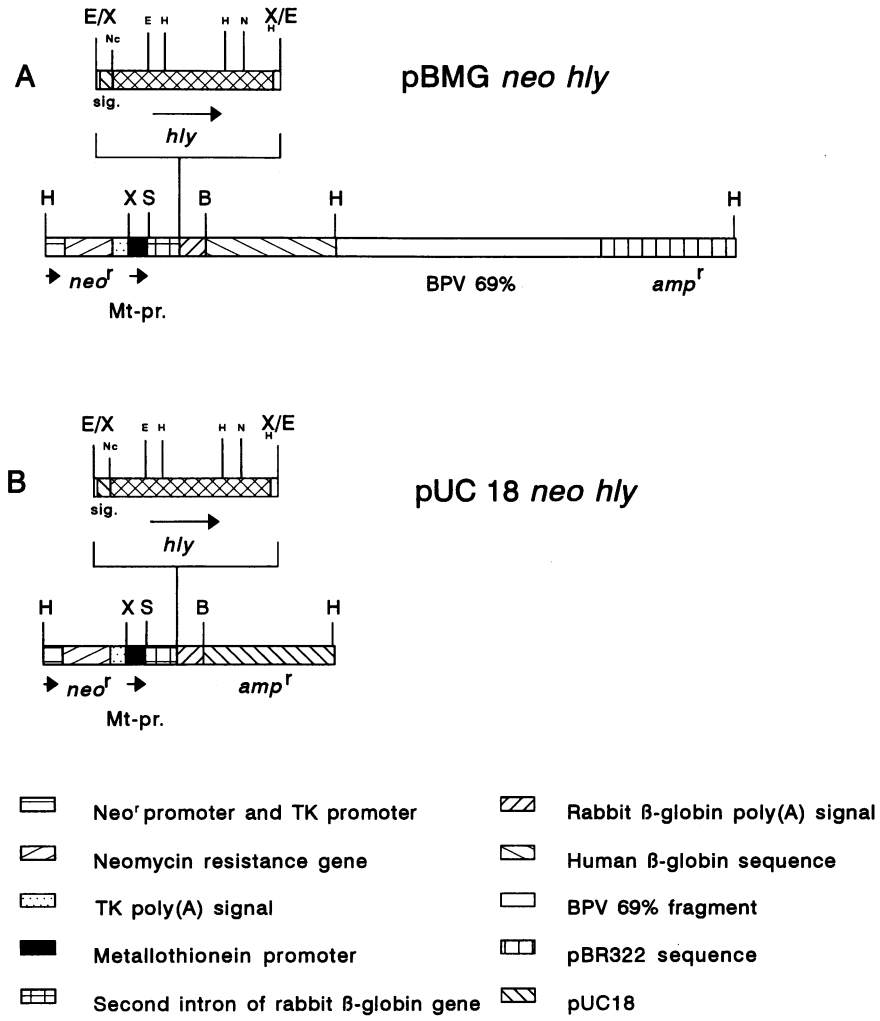

FIG. 1. Construction of the plasmids carrying the intact or leaderless *hly* gene of *L. monocytogenes* EGD Sv 1/2a. (A) Insertion of the *hly* gene into the expression vector pBMG *neo*. The amino-terminal signal sequence (sig.) can be deleted by using the *Nco*I site. (B) Insertion of the neomycin and listeriolysin expression cassettes of pBMG *neo hly* and pBMG *neo* ΔS *hly*, respectively, into pUC18. Restriction sites indicated are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Nco*I (Nc), *Sal*I (S), and *Xba*I (X). TK, thymidine kinase; BPV, bovine papillomavirus; Mt-pr, metallothionein promoter.

listeriolysin, whereas the leaderless listeriolysin could be detected.

Alterations in the cell morphology of the *hly*-transfected cells. To determine what effects expression of listeriolysin had in eukaryotic cells, the cell morphologies of the transfected cells were examined. 3T6 fibroblasts and L2 epithelial cells, transfected with the *hly* plasmids but grown without $ZnSO_4$, showed little alteration in growth and cell morphology when compared with untransfected cells or cells which were transfected with similar plasmids carrying the ovalbumin (*ova*) gene instead of the *hly* gene.

However, $ZnSO_4$ induction of the *hly* genes in transfected 3T6 and L2 cells led to dramatic changes in cell morphology and growth. Under the induced conditions, large cell aggregates which consisted of roundup cells were formed (Fig. 5). The formation of these cell foci was not caused by the pBMG vector, since the pUC18-derived construct exhibited the same effect. In addition, such foci were not formed in cells transfected with the pBMG construct carrying the *ova* gene with or without $ZnSO_4$ induction. The effect was also not caused by $ZnSO_4$, since treatment of both untransfected cell lines with various concentrations of $ZnSO_4$ did not alter the cell morphology.

The contact between the cells in a focus was very tight and could be dissolved neither by mechanical forces nor by treatment with trypsin or collagenase. Electron microscopic pictures of a representative cell aggregate showed that the peripheral cells of the aggregate appeared to be intact, while those in the center of the aggregate were severely damaged (Fig. 6).

Interestingly, 3T3 mouse fibroblasts, which in contrast to the 3T6 and L2 cells are contact inhibited, did not show formation of these cell aggregates when these cells were transfected with either pBMG- or pUC18-based *hly* constructs (data not shown).

3T6 cells transfected with the leaderless *hly* gene exhibited cytopathic effects. Their growth rate was slowed down, the cell layer never reached confluency, and the fibroblasts rounded up after only a few rounds of replication (Fig. 5). The formation of cell aggregates, a typical phenotype of the *hly*-transfected

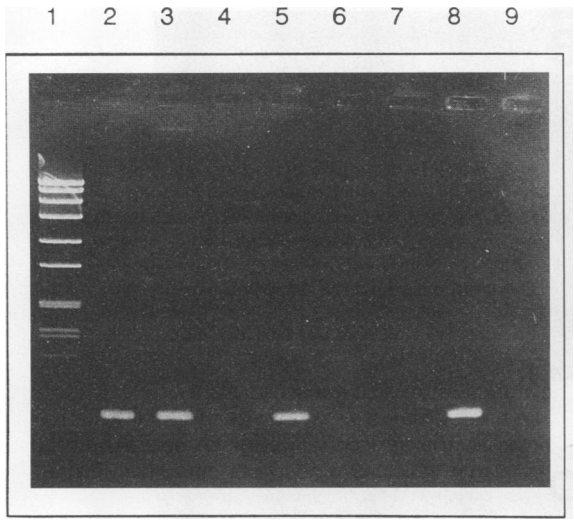

FIG. 2. PCR detection of the *hly* gene in transfected 3T6 mouse fibroblasts. The carboxy-terminal 600-bp fragment of the *hly* gene could be detected only in the Hirt extract of cells transfected by pBMG *neo* derivatives, whereas a positive signal was obtained only in the fraction of high-molecular-weight DNA of cells transfected by pUC18 derivatives. Lanes: 1, molecular weight marker; 2, *L. monocytogenes* EGD Sv 1/2a lysate; 3, 100 ng of pBMG *neo hly*; 4, pBMG *neo hly*-transfected 3T6 cell high-molecular-weight DNA; 5, pBMG *neo hly*-transfected 3T6 cell Hirt extract; 6, pBMG *neo ova*-transfected 3T6 cell high-molecular-weight DNA; 7, pBMG *neo ova*-transfected 3T6 cell Hirt extract; 8, pUC 18 *neo hly*-transfected 3T6 cell high-molecular-weight DNA; 9, pUC18 *neo hly*-transfected 3T6 cell Hirt extract.

3T6 cells, was never observed with these cells. L2 cells transfected with the leaderless *hly* gene did not show obvious differences in the cell morphology or the growth behavior compared with those of the untransfected cells. L2 cells were also less sensitive to externally added listeriolysin than were 3T6 cells. In cytotoxicity assays, this epithelial cell line proved to be more resistant to added listerial culture supernatant than 3T6 fibroblasts (Table 1). L2 cells transfected with the intact *hly* gene formed large piles of spherical cells after induction with $ZnSO_4$ (Fig. 5). Fluorescence microscopy revealed that under the induced conditions, the *hly* gene in transfected 3T6 cells led to significantly increased formation of actin microfilaments (Fig. 7).

Cell proliferation of *hly*-transfected cells is enhanced. The growth behavior of the *hly*-transfected 3T6 and L2 cells already suggested an enhanced growth of these cells compared with

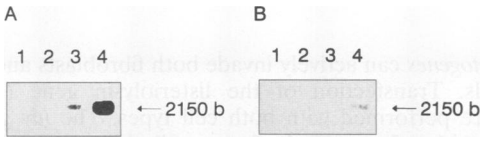

FIG. 3. Northern blot analysis of *hly* transcripts. Each lane contained 10 μg of total RNA from transfected 3T6 fibroblasts. After hybridization with a labeled *hly* probe (described in Materials and Methods), *hly* transcripts were detected by autoradiography. (A) Lanes 1 and 2, RNA of pBMG *neo ova*-transfected cells; lanes 3 and 4, RNA of pBMG *neo hly*-transfected cells that were noninduced or induced with 90 μM $ZnSO_4$ for 12 h, respectively. (B) Lanes 1 and 2, RNA of pUC18 *neo ova*-transfected 3T6 cells; lanes 3 and 4, RNA of pUC18 *neo hly*-transfected 3T6 cells that were noninduced or induced with 90 μM $ZnSO_4$ for 12 h, respectively.

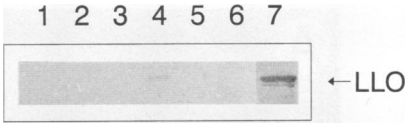

FIG. 4. Immunodetection of listeriolysin (LLO) in Triton X-100-lysed cell extracts of transfected 3T6 mouse fibroblasts by pBMG *neo* derivatives. The blot was probed with polyclonal antilisteriolysin antibody. Lanes: 1, pBMG *neo ova*-transfected 3T6 cells, noninduced; 2, pBMG *neo ova*-transfected 3T6 cells induced with 90 μM $ZnSO_4$; 3, 3T6 pBMG *neo* ΔS *hly* noninduced; 4, 3T6 pBMG *neo* ΔS *hly*-transfected 3T6 cells induced with 90 μM $ZnSO_4$; 5, pBMG *neo hly*-transfected cells, noninduced; 6, pBMG *neo hly*-transfected cells induced with 90 μM $ZnSO_4$; 7, $(NH_4)_2SO_4$-precipitated supernatant from *L. monocytogenes* NCTC 7973 1/2a.

the nontransfected cells. To obtain more quantitative data on the proliferation of the transfected cells, we determined the incorporation of radiolabeled thymidine in these cells under conditions of induced *hly* gene expression, i.e., in the presence of 90 μM $ZnSO_4$. As shown in Fig. 8, there is a significantly higher incorporation of radioactive thymidine in *hly*-transfected L2 cells than in the *ova*- or ΔS *hly*-transfected cells. Similar results were obtained when viable cell counts of 3T6 cells were determined over a longer period of time (Fig. 8). Interestingly, *hly*-transfected 3T3 cells, which did not generate cell aggregates when grown in culture, also showed a significantly enhanced proliferation compared with the nontransfected or *ova*-transfected cells (Fig. 8).

DISCUSSION

Listeriolysin is an essential virulence factor of *L. monocytogenes*, and synthesis of this cytolysin occurs in all clinical *L. monocytogenes* isolates. Recent studies have shown that this secreted protein is required for the release of the bacteria from the phagosomal compartment into the cytoplasm (8, 25, 39). It is assumed that the pore-forming property of listeriolysin is responsible for this step. Within eukaryotic cells, *L. monocytogenes* also has to dissolve the double membrane which is formed when the bacteria enter neighboring cells (cell-to-cell spread), so listeriolysin is probably necessary for this step as well. Both membrane disruption processes seem to occur in combination with phospholipases (12, 48). While the disruption of the phagosomal membrane is thought to be assisted by the phosphatidylinositol-specific phospholipase C (PlcA), the phosphatidylcholine-specific phospholipase C (PlcB) seems to be essential for the disruption of the double membrane (12, 48). Listeriolysin faces a membrane that is outside-in after initial uptake into eukaryotic cells, whereas the cytolysin encounters an inside-out orientation after cell-to-cell spread. Cholesterol-containing parts of the membrane seem to be receptors for listeriolysin, and only cholesterol-containing membranes are disrupted by this bacterial cytolysin (42). The distribution of cholesterol on both sides of the cytoplasmic membrane is probably not equal, and the accessibility for listeriolysin and therefore the efficiency of pore formation from both sides may not be the same. This could explain the strict requirement for PlcB in the disruption of the double membrane during cell-to-cell spread.

Besides pore formation, listeriolysin, like most other cytolysins, is a potent trigger of membrane-associated cascades (5, 10, 36). It has been shown that purified listeriolysin and bacteria expressing listeriolysin trigger the synthesis and the release of certain leukotrienes (26). Furthermore, listeriolysin-positive but not listeriolysin-negative *L. monocytogenes* strains

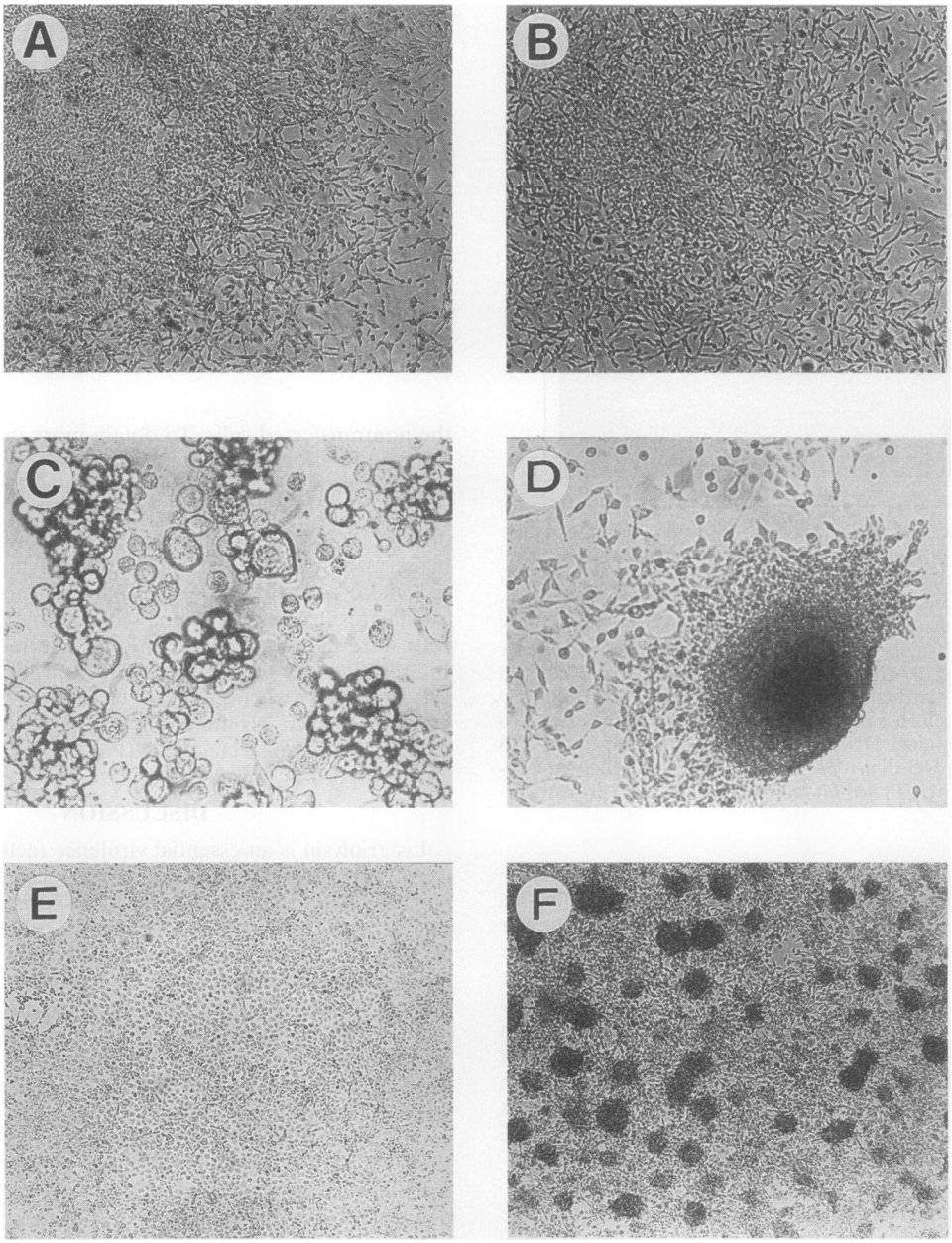

FIG. 5. Cell morphology of nontransfected (A), pBMG *neo ova*-transfected (B), pBMG *neo* ΔS *hly*-transfected (C), and pBMG *neo hly*-transfected (D) 3T6 mouse fibroblasts and nontransfected (E) and pBMG *neo hly*-transfected (F) L2 epithelial cells. Magnifications, ×320 (A, B, E, and F), ×480 (C), and ×350 (D).

cause the induction of cytokines such as interleukin 1α, tumor necrosis factor alpha, and interleukin 6 in macrophages (2, 28).

Most studies concerning cellular events in mammalian cells which are caused by listeriolysin have been performed with the entire *L. monocytogenes* system. However, inside the host cell various virulence factors interact in a complex way (13, 35). This makes it generally difficult to unravel all contributions of a single virulence factor to the effects observed in an infected host cell. The transfection of the bacterial gene encoding the virulence factor and its expression in the host cell's cytoplasm may help us to better understand such effects.

This approach was successfully applied to listeriolysin in this study. Previous investigations (19, 27) have shown that *L. monocytogenes* can actively invade both fibroblasts and epithelial cells. Transfection of the listeriolysin gene (*hly*) was therefore performed with both cell types. The *hly* gene was cloned under the control of a metallothionein promoter to allow the conditional expression of listeriolysin upon induction with $ZnSO_4$, avoiding possible deleterious effects for the mammalian cells when produced constitutively. Stable *hly* transfectants of all cell lines used (3T6 and 3T3 fibroblast as well as epithelial L2 lines) were obtained by using either a papillomavirus vector (pBMG *neo*) or the bacterial pUC18 plasmid for the delivery of the *hly* gene. As expected, the *hly* gene was found in an episomal state or integrated into the host cell's genome when pBMG *neo* or pUC18, respectively, was

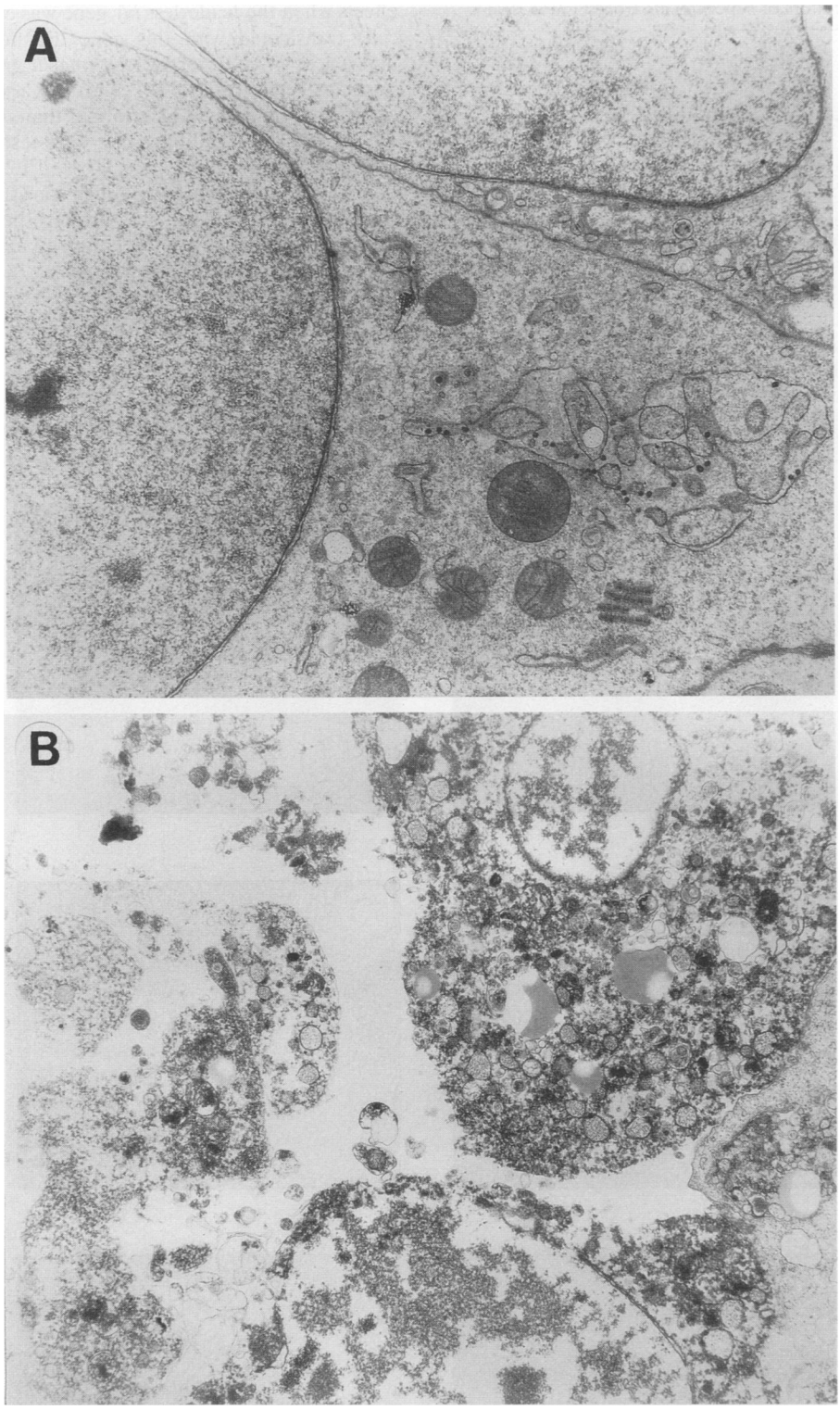

FIG. 6. Electron microscopy of pBMG-*neo* *hly*-transfected epithelial L2 rat cells showing the peripheral (A) and the central (B) regions of a cell aggregate.

utilized as a vector system. Addition of $ZnSO_4$ clearly transcriptionally induced *hly* gene expression. In the uninduced state, none of these transfectants exhibited abnormal behavior with respect to cell morphology or growth. Surprisingly, all *hly*-transfected cell lines showed a significantly enhanced proliferation when expression of the *hly* gene was induced by $ZnSO_4$. In addition, increased formation of actin microfilaments in these cells was observed. These effects were not

TABLE 1. Cytolytic titers of supernatants from *Listeria* strains

Strain	Cytolytic titer[a] against cell line:	
	3T6	L2
L. innocua 6b		
L. monocytogenes EGD 1/2a	4	2
L. monocytogenes NCTC 7973	8	4

[a] Hemolytic titer is expressed in complete hemolysis units, which are defined as the reciprocal of the highest dilution at which cytolysis of all cells was observed.

observed in *ova*-transfected cells with or without treatment with $ZnSO_4$ or in cells transfected with a *hly* gene that lacked the 5′ sequence encoding the N-terminal transport signal of listeriolysin. In fact, 3T6 cells showed significant cytopathic effects when the leaderless *hly* gene was expressed, whereas L2 cells transfected with this gene did not exhibit detectable differences in cell morphology or cell growth compared with nontransfected cells. 3T6 cells are also more sensitive to extracellularly applied listeriolysin than are L2 cells.

While all three cell lines that were tested also exhibited the enhanced proliferation, only 3T6 and L2 cells showed formation of large cell aggregates, which consisted of densely packed spherical cells. The contact between these cells is extremely tight and was not disrupted either by mechanical force or by treatment with trypsin or collagenase.

Our data clearly show that listeriolysin must be responsible for the enhanced cell proliferation and the formation of the cell aggregates, because (i) both effects were observed only upon induction of *hly* gene expression with $ZnSO_4$, (ii) $ZnSO_4$ alone in the same concentration as applied for induction had no effect on any of the cell lines used, and (iii) both effects were

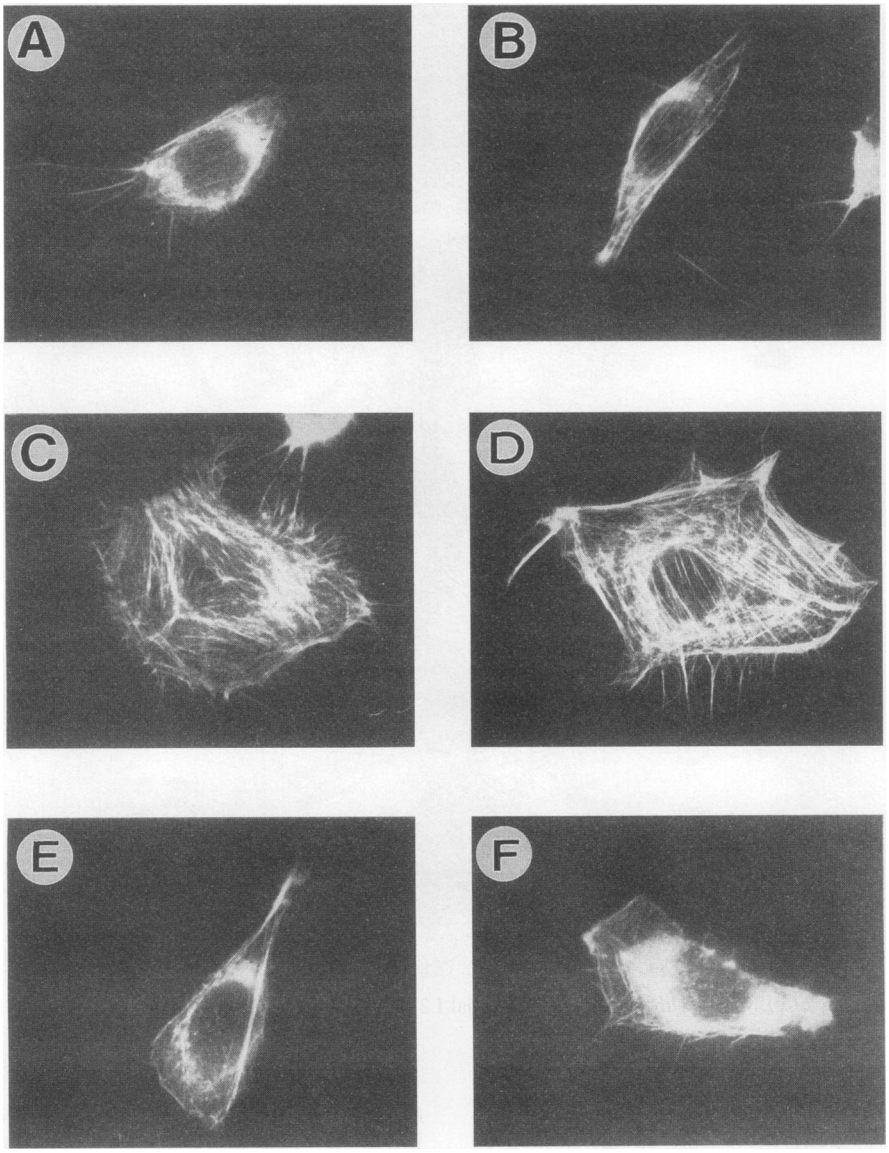

FIG. 7. Phalloidin staining of actin stress fibers of nontransfected (A and B), pBMG *neo hly*-transfected (C and D), and pBMG *neo ova*-transfected (E and F) 3T6 mouse fibroblasts.

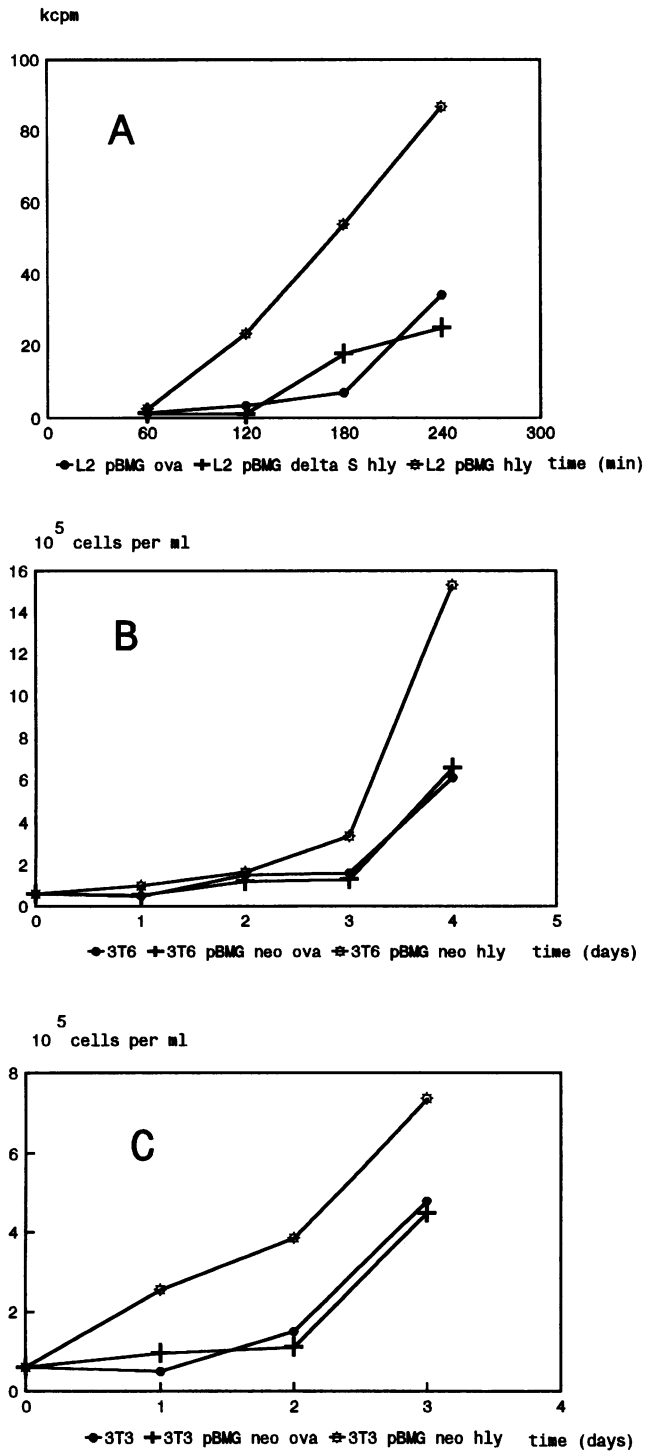

FIG. 8. Determination of proliferation rate of *hly*-transfected and nontransfected cells. (A) [^{3}H]thymidine incorporation in L2 cells transfected with pBMG *neo* derivatives; (B and C) viable cell counts of 3T6 (B) and 3T3 (C) cells transfected with pBMG *neo* derivatives.

obtained regardless of whether the *hly* gene was introduced into these cells by the papillomavirus vector pBMG *neo* or by the bacterial plasmid pUC18. In addition, the pBMG vector alone or pBMG carrying the ovalbumin gene instead of the *hly* gene did not exert these effects in transfected cells.

Since only listeriolysin carrying the transport signal sequence enhances cell proliferation, we postulate that the bacterial protein enters the protein secretion pathway of the mammalian cell and ends up in the cytoplasmic membrane. There it may induce signal transduction of as yet unknown cascades, which could ultimately lead to enhanced cell proliferation. It has been recently shown that listeriolysin leads to mitogen-activating protein kinase activation and to activation of protein kinase C in eukaryotic cells (15, 26). The putative transport of listeriolysin into the cytoplasmic membrane may also be the reason why we never detected listeriolysin by immunoblotting or immunoprecipitation with extracts of the induced cells. Proteolytic degradation and/or glycosylation of listeriolysin may occur in the membrane compartment, which may inhibit binding to the antibodies. This assumption is supported by the detection of listeriolysin when the cells were transfected with the leaderless *hly* gene. This protein, which is expressed by the same promoter as the intact listeriolysin, apparently remains rather stable in the cytoplasm. Inside the cytosol it may act on membranes of cellular organelles, potentially causing the cytopathic effects observed, especially in 3T6 cells.

In contrast to bacterial cytotoxins like exotoxin A of *Pseudomonas aeruginosa* and invasive adenylate cyclase of *Bordetella pertussis*, which kills mammalian cells when expressed intracellularly (50), bacterial cytolysins such as aerolysin of *Aeromonas hydrophila* (3), alpha-toxin of *Staphylococcus aureus* (49), and the listeriolysin that was studied here do not kill the cells. Enhancement of cell proliferation was observed only when listeriolysin was expressed intracellularly. Listeriolysin is also the only cytolysin among the described cytolysins which is produced by an intracellular microorganism. As described above, listeriolysin is synthesized in the phagosomal compartment, where it acts by disrupting the phagosomal membrane. Since listeriolysin is a pore-forming protein, it is probably inserted into the phagosomal membrane during this stage and presumably is recycled together with residual phagosomal membrane fragments. These recycled membranes could be integrated into the cytoplasmic membrane of the infected cell, possibly triggering proliferation of the infected cell. This would generate additional host space for the invasive microorganism and hence provide a significant advantage to the infecting microorganism.

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