INTRODUCTION

The cholesterol-dependent cytolysin (CDC) produced by the food-borne, facultative intracellular pathogen *Listeria monocytogenes* is known as listeriolyisin O (LLO). Its coding gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria*. Subsequent characterization of the *hly* locus led to the discovery of *Listeria* pathogenicity island 1 (LIPI-1), a 9-kb chromosomal gene cassette encoding key functions necessary for the intracellular parasitic lifestyle of these bacteria. The elucidation between 1986 and 1989 of the crucial role that LLO plays in escape from the phagocytic vacuole made this toxin the first microbial product for which a function critical for the survival and replication of a parasite within host cells was identified. There is growing evidence that LLO is a multifaceted virulence factor with many other, sometimes subtle roles in the host-pathogen interaction. LLO displays all the common features of CDCs, but also unique properties making it a particularly interesting member of this toxin family. This chapter reviews the advances made in our understanding of the CDC with the best characterized role in pathogenesis. It also illustrates how this fundamental knowledge can be exploited to harness a nasty microbial product for the development of novel vaccines and therapeutic tools.

CHARACTERIZATION

Early studies

The production of a soluble hemolysin by *L. monocytogenes* was first reported by Harvey and Faber in 1941. During the 1960s and 1970s, various groups attempted the purification and characterization of this activity (Girard et al., 1963; Jenkins and Watson, 1971; Njoku-Obi et al., 1963; Siddique et al., 1974; Watson and Lavizzo, 1973). Jenkins et al., (1964) showed for the first time that the listerial hemolysin is functionally and antigenically related to streptolysin O (SLO), and Kingdon and Sword (1970) demonstrated two of its key characteristics: inhibition by cholesterol and pH optimum below 7. These authors also suggested that the *L. monocytogenes* hemolysin might be involved in the disruption of phagosomal membranes. Finally, Geoffroy et al. (1987) purified the toxin to homogeneity, demonstrated that it was a member of the CDC family, named it LLO, and determined that it was active at a narrow pH range, between 4.5 and 6.5 with optimum at 5.5. The 58-kDa CDC from *Listeria ivanovii*, ivanolysin O (ILO), was later purified and characterized (Vázquez-Boland et al., 1989a), and it was shown that the weakly hemolytic but non-pathogenic species *Listeria seeligeri* also produced an LLO-related CDC, albeit in small amounts (Geoffroy et al., 1989, Leimeister-Wächter et al., 1989).

Molecular genetic studies

The *hly* gene encoding LLO was first identified and sequenced by Mengaud et al. (1988). Analysis of its deduced sequence and that of the cloned genes for ILO and the *L. seeligeri* hemolysin, seeligerilysin O (LSO) (Haas et al., 1992), revealed that the listerial hemolysins, although similar, are not identical (Figure 40.1). Their degree of similarity, 76–82% and 77–78% identity at the amino acid and nucleotide levels,
respectively, is comparable to that found between other orthologues shared by the three listerial species, consistent with the view that the central virulence gene cluster LIPI-1 bearing the hemolysin determinant (Figure 40.2) evolved vertically during speciation of the common Listeria ancestor (Vázquez-Boland et al., 2001a; Schmid et al., 2005).

STRUCTURE-FUNCTION

CDC structure and mechanism of pore formation

A crystal structure is available for the soluble monomer of perfringolysin O (PFO) (Rossjohn et al., 1997). The extensive overall similarity of CDCs in terms of amino-acid sequence and functional properties, it is reasonable to assume that the domain structure of PFO can be extrapolated to any member of this toxin family. Figure 40.3 depicts the theoretical three-dimensional structure of LLO modeled on the basis of the crystallographic data for PFO. LLO is predicted to consist of four discontinuous, predominantly β-sheet-folded domains spatially organized into two distinct protein regions: an N-terminal region formed by domains 1 to 3 and a C-terminal region formed by domain 4. Domains 1, 2, and 4 are aligned vertically, whereas domain 3 is located parallel to domain 2, forming an elongated mushroom-like molecule. The C-terminal domain 4 folds into a compact β-sheet sandwich that is linked to the N-terminal region via residue Gly417 (Gly392 in PFO).

Membrane disruption by CDCs involves the transition from monomeric, water-soluble toxin molecules to non-covalently bound oligomeric, insoluble ring-shaped toxin structures formed by up to 50 monomers, which insert into the membrane, forming large pores about 20 to 30 nm in diameter (Bhakdi et al., 1985; Alouf and Geoffroy, 1991; Morgan et al., 1994; Sekiya et al., 1993) (Figure 40.4). A clear picture of the cytolytic mechanism of CDCs has begun to emerge from the integration of PFO structural data, site-specific mutagenesis analyses, and the information provided by bioophysical and fluorescent probe-based studies performed with SLO, pneumolysin (PLY), and particularly PFO (Rossjohn et al., 1997; Nakamura et al., 1998; Palmer et al., 1998a, 1998b; Shepard et al., 1998, 2000; Gilbert et al., 1999; Shatursky et al., 1999; Heuck et al., 2000, 2003; Ramachandran et al., 2002, 2004) (see relevant chapters in this book for more information). These studies suggest that domain 4 mediates membrane binding and domains 1–3 are involved in toxin oligomerization and membrane disruption. This is consistent with experimental data obtained with LLO showing that neutralizing antibodies against the native toxin that map to domain 4 inhibit binding to cellular membranes (De los Toyos et al., 1996), whereas neutral-
izing epitopes that do not inhibit binding to membranes map to domain 1 (Darji et al., 1996).

In the consensus model, initial membrane contact via domain 4 (Heuck et al., 2000; Ramachandran et al., 2002) would facilitate the interaction of toxin molecules with cholesterol. This would trigger a conformational change in the monomer, increasing its capacity to self-aggregate and, via the refolding of three α-helices of domain 3 into two membrane-spanning β-hairpins, its hydrophobicity and membrane affinity (Shatursky et al., 1999). By lateral diffusion, the vertically arranged membrane-bound monomers would collide with other monomers to form an oligomeric prepore (Shepard et al., 2000; Hotze et al., 2002; Heuck et al., 2003) via edge-to-edge association of domain 3 β-strands (Ramachandran et al., 2004). In a second step, the prepore would insert into the membrane by vertical collapse of the toxin structure, which would make the β-hairpins extend across the bilayer, forming a transmembrane β-barrel pore (Czajkowsky et al., 2004). The notion that membrane binding and disruption are dissociable events in CDC function is supported by experiments with LLO showing that monoclonal antibodies that neutralize cytolytic activity do not affect toxin binding to target membranes (Nato et al., 1991; Darji et al., 1996). This is possible if these antibodies block downstream events of toxin fusion, such as oligomerization or pore formation.

Domain 4

Most targeted mutagenesis studies have focused on domain 4, as this region contains the conserved C-terminal Trp-rich motif, ECTGLAWEWWR, a hallmark of this toxin family. As in other CDCs (Pinkney et al., 1989; Saunders et al., 1989; Sekino-Suzuki et al., 1996; Baba et al., 2001), mutational analyses in LLO have shown that this domain, in particular the conserved motif and the C-terminus, is critical for activity (Mengaud et al., 1988; Kohda et al., 2002). In LLO, single amino-acid substitutions in the undecapeptide resulted in L. monocytogenes strains with various degrees of attenuation proportional to the loss of cytolytic activity (Michel et al., 1990). LSO from the non-pathogenic L. seeligeri carries a naturally occurring Ala-to-Phe substitution in the undecapeptide (Haas et al., 1992) (Figure 40.1), which has been shown to be responsible for the weak cytolytic activity for sheep erythrocytes exhibited by this toxin (Ito et al., 2001).

Role of the single Cys residue

The undecapeptide motif contains the Cys residue that mediates the characteristic reversible inhibition by oxidation—and reactivation by thiol-reducing agents—that gave this toxin family its former name, “oxygen-labile” or “thiol-activated” hemolysins. The activating effect of thiol-reducing agents was initially thought to be due to the breakage of an intramolecular bond, but sequencing of the LLO gene and of other CDC genes revealed that in most cases only one Cys residue is present in the toxin molecule. This residue was also thought to be essential for activity and to be involved in cholesterol binding (see below) (Smyth and Duncan, 1978; Alouf and Geoffroy, 1991; Alouf, 1999). However, although replacement of the Cys residue by Ala in LLO rendered the toxin insensitive to
inactivation by oxidation or thiol-alkylating agents, it had no significant effect on cytolytic activity (Michel et al., 1990). Similar results were obtained with other toxins of the family (Pinkney et al., 1989; Saunders et al., 1989).

The non-essential role of the Cys residue raises questions as to why it is subject to such pressure for conservation. In LLO and other CDCs, its replacement by bulkier residues abolishes most of the activity, suggesting that the mechanism underlying the Cys-mediated reversible inhibition of cytolytic activity involves steric hindrance (Pinkney et al., 1989; Saunders et al., 1989; Michel et al., 1990). Such an effect may result from the formation of toxin dimers or heterodimers with other proteins via disulfide bridges. Indeed, ILO copurifies with a major 27-kDa protein of the L. ivanovii culture supernatant, and this heterodimer is resolved by treatment with thiol-reducing agents (Vázquez-Boland et al., 1989a). This 27-kDa protein is another listerial virulence factor, the small/excreted internalin i-InlE (Engelbrecht et al., 1998), the mature form of which contains a single Cys. This suggests that the Cys residue present in the undecapeptide of most CDCs may play a role in the targeting of other virulence-associated proteins to the eukaryotic cell membrane. It may also have a regulatory role in vivo, for example, by enhancing toxin activity in low-pH (reducing) environments, such as the acidified vacuolar compartment.

Cholesterol binding

Based on the capacity of cholesterol to inhibit the cytolytic activity of CDCs irreversibly and the fact that these toxins are active only against cholesterol-containing membranes, it is generally accepted that this membrane lipid acts as the toxin receptor (Smyth and Duncan, 1978; Alouf and Geoffroy, 1991). Indeed, many studies have shown that the extent of CDC interaction with the membrane is directly dependent on the amount of cholesterol present on that membrane (Jacobs et al., 2001; Heuck et al., 2000; Waheed et al., 2001), and a cholesterol-binding region has been unambiguously traced to domain 4 in PFO (Shimada et al., 1999, 2002). Consistent with this, C-terminal truncations in LLO, whether involving the undecapeptide or not, abolished binding to immobilized cholesterol (Kohda et al., 2002). Experiments with recombinant PLY polypeptides also showed that domain 4 alone binds free cholesterol, whereas a truncated protein...
comprising only domains 1–3 does not (Baba et al., 2001). In some of these studies, the abolition of cholesterol-binding activity was associated with a loss of membrane-binding activity (Shimada et al., 1999; Baba et al., 2001), in line with the view that cholesterol recognition is essential for initial interaction with the membrane.

However, experiments in which preincubation of LLO with cholesterol did not impede the binding of the toxin-cholesterol complexes to the cell membrane (Jacobs et al., 1998) challenged the above notion. A similar dissociation of cholesterol- and membrane-binding activities was observed in experiments using PFO mutants with single Phe substitutions in the three Trp residues of the undecapeptide. These mutations impaired membrane binding, but did not affect cholesterol binding (Sekino-Suzuki et al., 1996). The observation that CDCs in solution form highly amphiphilic complexes with free cholesterol led to the development of a simple one-step purification method by selective precipitation with cholesterol (Vázquez-Boland et al., 1989a). Using this technique, an inactive, truncated LLO lacking the 49 C-terminal residues (i.e., approx. half of domain 4, including the entire conserved undecapeptide) (Mengaud et al., 1988) was shown to form cholesterol-toxin complexes (Vázquez-Boland et al., 1989b). Thus, domains involved in cholesterol binding appear to be present outside the undecapeptide and C-terminus of the toxin, either in the remaining part of domain 4 (a possibility not supported by recent evidence obtained with fluorescent probes showing that, except for its tip—the Trp-rich loop—all parts of domain 4 in membrane-bound PFO are accessible to the solvent; Heuck et al., 2000) or in the N-terminal region. The latter option is consistent with the observation that although the cholesterol-complexed LLO binds to target membranes, there is no oligomerization (Jacobs et al., 1998), suggesting that preincubation with cholesterol blocks sites involved in pore formation (which reside in the N-terminal region; see above). Moreover, the above-mentioned PFO mutants that showed reduced membrane-binding and cytolytic capacities while retaining cholesterol-binding activity were still able to form ring-shaped structures on membranes (Sekino-Suzuki et al., 1996). These observations suggest that, rather than acting as the toxin receptor, cholesterol might play a critical role in subsequent stages of membrane-toxin interaction, i.e., during oligomerization and/or membrane insertion.

The view that cholesterol’s primary role is to facilitate membrane insertion has received strong support from recent studies showing that cholesterol-depleted membranes stalled PFO, SLO, and intermedilysin (ILY, from Streptococcus intermedius) at the prepore stage. All three toxins regained lytic activity if cholesterol was restored to the membrane, suggesting that this lipid is critically involved in the prepore to transmembrane pore transition (Giddings et al., 2003).

Role of the conserved undecapeptide

The conserved ECTGLAWEWWR undecapeptide forms a hydrophobic loop at the base of domain 4 (Figure 40.3). This structure penetrates the lipid bilayer and is thought to be responsible for the initial attachment of the CDC monomers to the target membrane during prepore formation (Heuck et al., 2000; Ramachandran et al., 2002). However, its exact role in toxin function remains unclear, as the contradictory data obtained with LLO illustrate. Thus, membrane binding was not affected in LLO mutants with impaired cytolytic activity due to amino-acid replacements in the undecapeptide, including Trp to Ala substitutions, which almost totally abolished toxin function (Michel et al., 1990). Similarly, monoclonal antibodies produced against a synthetic LLO undecapeptide and that recognized the immobilized toxin did not inhibit membrane binding or cytolysis (Nato et al., 1991). In contrast, a neutralizing monoclonal antibody raised against native toxin and that specifically bound to the undecapeptide caused LLO membrane-binding inhibition and no longer recognized its target epitope in a pre-formed toxin/membrane complex (Jacobs et al., 1999). The latter experiments are consistent with results obtained with the above-mentioned weakly hemolytic PFO mutants containing Phe-to-Trp substitutions in the undecapeptide, as these displayed significantly impaired erythrocyte membrane-binding activities (Sekino-Suzuki et al., 1996). Circular dichroism analyses of membrane-associated mutant PFO toxins suggested that both Trp438 and Trp439 were involved in a conformational change occurring during pore formation (Nakamura et al., 1998). Thus, the impaired interaction with the membrane seen with the PFO mutants or LLO bound to the undecapeptide-specific antibody may indicate a role for the conserved motif in the adoption of the correct folding required for membrane insertion and pore formation, rather than a role in the initial stages of toxin-membrane interaction.

Such an interpretation is consistent with the observed inhibition of PLY self-interaction by derivatization of the single Cys residue with the thiol-active agent dithio(bis)nitrobenzoic acid (Gilbert et al., 1998).

Recently, PFO and ILY mutants displaying a significant loss of hemolytic activity, but retaining wild-type levels of membrane-binding activity, have been shown to oligomerize on the membrane but to be unable to form a pore complex, consistent with a role for the undecapeptide in the prepore-to-pore conversion.
This study also showed that target cell recognition by ILY depends on domain 4, but does not involve the undecapeptide (Polekhina et al., 2005) (see below).

Domains 1–3

A recent study with independently expressed N-terminal (domains 1–3) and C-terminal (domain 4) LLO polypeptides has shown that the two protein fragments were able to bind to erythrocyte membranes and intact mammalian cells, the former even with greater affinity (Dubail et al., 2001). Intriguingly, functional complementation was reported to occur after co-production of both LLO subfragments in L. monocytogenes, suggesting that they can reassemble to form a pore complex (Dubail et al., 2001). Also intriguing was that a Δhly L. monocytogenes strain, trans-complemented with a plasmid encoding a truncated toxin variant lacking the first three residues of the native protein, displayed similar levels of hemolytic activity to wild-type L. monocytogenes in vitro, but apparently disrupted the phagocytic vacuole less efficiently and was two orders of magnitude less virulent in the mouse model (Lety et al., 2003). It is unclear whether this was actually due to the lack of these three first residues in LLO, which would imply that they play a specific role in the interaction with the phagosomal membrane, or to problems of in vivo instability of the plasmid construct encoding the truncated LLO toxin.

ROLE IN INTRACELLULAR PARASITISM

Cell biology of Listeria infection

Listeria belong to a subgroup of intracellular bacterial pathogens that replicate in the cytosol and not in a membrane-bound vacuole. Soon after phagocytosis, whether by macrophages or by any other susceptible cell type actively invaded by these bacteria (epithelial cells, hepatocytes, endothelial cells, etc.), listeriae are seen free in the cytosol. Intracytosolic bacteria polymerize host cell actin at one of their poles by means of the surface protein ActA, a mechanism that propels them across the cytosol at a speed of 0.3 µm/s. The movement is random, and some bacteria eventually reach the cell periphery and protrude from the cell surface at the tip of pseudopod-like evaginations, which penetrate into neighboring cells. This triggers a phagocytosis process, resulting in the formation of a “secondary” phagosome that is surrounded by a double membrane, from which Listeria escape again to begin a new replication cycle (Figure 40.5). Thanks to this direct cell-to-cell spread mechanism, Listeria rarely leave the intracellular host compartment, facilitating avoidance of the humoral effectors of the immune system and phagocytosis by migrant neutrophils. By rapidly spreading to neighboring cells, they escape the cellular immune response typically directed against infected cells displaying cytosol-released bacterial epitopes in association with MHC class I molecules (San Mateo et al., 2002). This strategy contrasts with that used by many other intracellular pathogens (e.g., Salmonella or Mycobacterium), which avoid the cellular immune response by hiding inside a vacuole, an enclosed environment that prevents/delays the presentation of antigens via the cytosolic MHC class I pathway (for recent reviews see Vázquez-Boland et al., 2001b; Portnoy et al., 2002; Dussurget et al., 2004).

Efficient disruption of the phagocytic vacuole membranes is therefore a key process in Listeria pathogenesis.

Role of LLO in phagosome disruption

The pathogenic Listeria spp, L. monocytogenes and L. ivanovii, are hemolytic, whereas the non-pathogenic spp, L. innocua, L. welshimeri, and L. grayi, are non-hemolytic. The only exception to this rule is L. seeligeri, which is hemolytic but non-pathogenic. However, as mentioned above, the hemolysin from this species, LSO, is weakly active and is produced in minute amounts (see below). The strong correlation between hemolytic activity and pathogenicity in the genus Listeria led various groups to generate, in the mid-late 1980s, isogenic hemolysin mutants by transposon mutagenesis. Analysis of the insertion region in one of these mutants led to the identifica-
tion and characterization of the hly gene (Mengaud et al., 1987, 1988). The non-hemolytic mutants were much less virulent in mice (increase greater than 4 logs in LD<sub>50</sub>), their spontaneous revertants recovered full pathogenicity, and their trans-complementation with the hly gene restored virulence to wild-type levels (Gaillard et al., 1986; Kathariou et al., 1987; Portnoy et al., 1988; Cossart et al., 1989).

Cell culture–based infection assays with these mutants showed that LLO is required for the intracellular survival and proliferation of <i>L. monocytogenes</i> in macrophages and non-professional phagocytes (Gaillard et al., 1987; Kuhn et al., 1988; Portnoy et al., 1988). Electron microscopy of infected cells revealed that hly mutants remained entrapped within phagosomes, indicating that LLO mediates disruption of the phagosome membrane (Gaillard et al., 1987). This was elegantly confirmed by experiments in which the hly gene was expressed in <i>Bacillus subtilis</i>; this conferred on the non-pathogenic bacterium the ability to escape from the phagocytic vacuole and to replicate within host cells (Bielecki et al., 1990). LLO not only mediates lysis of the primary phagosomes formed after the uptake of extracellular bacteria, but it is also required for the efficient escape of <i>L. monocytogenes</i> from the double-membrane secondary vacuole that forms upon cell-to-cell spread (Gedde et al., 2000; Dancz et al., 2002).

Although LLO plays a critical role in phagosome disruption, three other membrane-damaging listerial proteins, the phospholipases C PlcA and PlcB, and the sphingomyelinase SmcL in <i>L. ivanovii</i>, cooperate in this process (see Vázquez-Boland et al., 2001b). The membrane lesions caused by LLO (Figure 40.4) probably facilitate the access of these enzymes to their substrates in the phagosomal membrane, leading to its total dissolution.

The CDC from <i>L. ivanovii</i>, ILO, plays a similar role in intracellular infection. Thus, like <i>L. monocytogenes</i> LLO<sup>+</sup> mutants, <i>L. ivanovii</i> ILO<sup>+</sup> mutants are strongly impaired in intracellular growth (Figure 40.6), and expression of the ILO gene in a Δhly mutant of <i>L. monocytogenes</i> complements the absence of LLO (Frehel et al., 2003). Even LSO from the non-pathogenic species <i>L. seeligeri</i>, albeit weakly active, appears to be able to promote phagosome disruption. The weakly hemolytic phenotype of this species is in part due to defective expression of the virulence gene (hly)-activator protein PrfA as a result of the insertion of a divergently transcribed ORF between the two genes of the <i>plcA-prfA</i> operon (Figure 40.2).

Complementation of <i>L. seeligeri</i> with a functional copy of this operon activated the expression of LSO and conferred to the bacterium the ability to escape from the phagosome and to replicate in the cytosol (Karunasagar et al., 1997).

**FIGURE 40.6** Intracellular growth of wild-type <i>L. ivanovii</i> and an isogenic ILO<sup>−</sup> mutant in bovine MDBK cells. As with <i>L. monocytogenes</i>, inactivation of the hly gene results in total loss of intracellular proliferation capacity in <i>L. ivanovii</i>.

**Adaptation of LLO to the intracellular niche**

Experiments in which the genes encoding SLO and PFO were expressed in <i>B. subtilis</i> showed that, despite their very close structural and functional similarities, not any CDC can supplant LLO’s role in intracellular parasitism. SLO did not facilitate intracellular growth, whereas PFO did; however, in contrast to what is observed with LLO, PFO-mediated growth was associated with host cell damage (Portnoy et al., 1992). Expression of the pfo gene also failed to restore virulence for mice in a <i>L. monocytogenes</i> hly mutant. The resulting strain was hemolytic and showed some ability to escape from phagosomes, but PFO damaged the host cells, thereby preventing normal intracellular growth (Jones and Portnoy, 1994). These results indicated that LLO, produced by an intracellular pathogen requiring an intact intracellular niche to replicate and to spread in host tissues, has evolved mechanisms for preventing cytotoxicity not found in PFO, originating from an extracellular pathogen associated with infections characterized by massive tissue destruction. PFO mutations resulting in a lack of cytotoxicity were identified by selecting for pfo-expressing <i>L. monocytogenes</i> mutants capable of normal intracellular growth. One type of mutation shifted the pH optimum of PFO (normally active in both acidic and neutral conditions) to a value similar to that for LLO, whereas the other decreased the cytosolic half-life of the toxin, providing
insight into the mechanisms by which LLO-mediated cytotoxicity is controlled (Jones et al., 1996).

Indeed, weak activity at pH 7.4 (similar to that of the eukaryotic cell cytosol) and a pH optimum of 5.5 (similar to that within phagosomes) is a clever strategy to restrict the membrane-damaging activity of LLO to the acidified vacuole, and thereby to prevent potential deleterious effects of toxin leakage into the cytosol. Evidence for such phagosome-specific compartmentalized activity of LLO has been provided by experiments using the membrane-impermeant fluorophore, 8-hydroxypyrene-1,3,6-trisulfonic acid, in murine macrophages. Rapid acidification of L. monocytogenes containing phagosomes was observed, followed by an increase in pH and the release of the dye from the vacuole. Loss of the fluorophore was prevented by lysosomotropic agents, such as ammonium chloride and bafilomycin A1 (Beauregard et al., 1997). These data are consistent with activation of LLO in the acidified phagosome sequentially leading to membrane permeabilization, pH equilibration, and the automatic deactivation of LLO. A single amino acid in domain 4, Leu461 (position 438 in the mature protein as shown in Figure 40.1), was identified as being responsible for the low optimum pH of LLO. Among CDCs, only listerial hemolysins have a Leu residue at this position. The replacement of Leu461 by Thr, as in PFO, caused a 10-fold increase in LLO activity at neutral pH, and L. monocytogenes carrying the mutant LLO toxin were cytotoxic (Glomski et al., 2002).

The short half-life of LLO in the cytosol is due to the presence in the N-terminus of a PEST-like sequence, normally involved in the targeting of eukaryotic proteins for cytosolic degradation. Deletion of this sequence from LLO increased cytotoxicity and lowered the virulence of the strain; conversely, introduction of the PEST sequence into PFO reduced cytotoxicity, and the mutant PFO toxin supported intracellular growth when produced in L. monocytogenes (Decatur and Portnoy, 2000).

The relevance of these distinctive features of LLO to pathogenesis is illustrated by a recent study in mice using L. monocytogenes strains with mutations that fail to compartmentalize toxin activity. The more cytotoxic the strain in cell culture, the less virulent it was for mice. The depletion of neutrophils, primarily involved in the killing of extracellular Listeria, and treatment with gentamicin, active only against extracellular bacteria, increased the relative virulence of the cytotoxic strain, reflecting that the control of LLO cytolytic activity is a key element in the avoidance of extracellular host defenses by L. monocytogenes (Glomski et al., 2003).

**III. TOXINS ACTING ON THE SURFACE OF TARGET CELLS (EXCEPT SUPERANTIGENS)**

**OTHER ROLES IN INFECTION**

**Immune response**

LLO is critically involved in the immune response to L. monocytogenes in several ways: (i) The LLO-mediated release of bacteria into the cytosol and subsequent intracellular growth are essential for MHC class I-restricted listerial antigen presentation and the induction of specific protective cytotoxic CD8+ T cells (Berche et al., 1987; Brunt et al., 1990; Barry et al., 1992; Hintbol et al., 1996). It has recently been reported that cytosolic listeriae activate type I IFN expression and many IFN-β-induced mutants, whereas phagosome escape-deficient (hly) mutants do not (Stockinger et al., 2002; McCaffrey et al., 2004). Non-pathogenic bacteria engineered to enter the host cytosol via heterologous hly expression also triggered this response (O’Riordan et al., 2002). Thus, via its membrane-disrupting activity, LLO may play a crucial role not only in the development of the acquired immune response, but possibly also in the activation of key innate immunity mechanisms. (ii) LLO is itself a major protective antigen recognized by Listeria-specific CD8+ cytotoxic T cells (CTLs) during infection (Berche et al., 1987a; Safley et al., 1991; Bouwer et al., 1992; Hess et al., 1996; Sirard et al., 1997). LLO is processed very efficiently into peptides that are presented by MHC class I molecules (Villanueva et al., 1995), and one such peptide, nonamer LLO 91–99, is an immunodominant epitope that induces specific CD8+ CTLs, which protect in vivo against L. monocytogenes infection and confer significant anti-Listeria immunity on naive mice upon passive transfer (Pamer et al., 1991; Harty et al., 1992). (iii) Pore formation by exogenous LLO has been shown to mediate the delivery of soluble antigens to the TAP-dependent cytosolic MHC class I antigen-presentation pathway (Darji et al., 1995, 1997), providing an additional mechanism for the generation of CD8+ CTLs against antigens secreted/released by extracellular L. monocytogenes. (iv) LLO elicits a potent humoral response and anti-LLO antibodies are detectable after infection with L. monocytogenes (see below). Administration of a murine anti-LLO neutralizing monoclonal antibody increased resistance to Listeria infection in mice (Edelson et al., 1999), thus challenging the old paradigm of cell-mediated immunity, according to which antibodies are not involved in protection against intracellular parasites (Kaufmann, 1993). It is therefore possible that the humoral arm of the immune system contributes to protective immunity against L. monocytogenes and that LLO also plays a key role in this protection. Finally, (v) through its modulin activity,
LLO may influence orchestration of the immune response to *Listeria* (see next section).

**Modulation of host responses**

LLO has been shown to induce a number of host-cell responses in a variety of cell types, indicating that this toxin is a versatile virulence factor, not only having a direct physical action on phagosome membranes, but also signaling effects on host cells that can influence the fate of *Listeria* infection. One of the mechanisms possibly involved is related to the ability of exogenous LLO to permeabilize cell membranes. Exogenous LLO at sublytic concentrations has been shown to produce Ca$^{2+}$-permeable pores, causing the influx of extracellular Ca$^{2+}$-ions into the cell (Repp *et al.*, 2002). This may enable cell surface-attached *L. monocytogenes* bacteria to modulate/enhance their entry into host cells, the key first step in listerial intracellular parasitism (Dramsi and Cossart, 2003). LLO-mediated permeabilization of the cell membrane is also required for the induction by the listerial phosphatidylinositol-specific phospholipase C (PlcA) of phosphoinositide hydrolysis and diacylglycerol (DAG) accumulation in human endothelial cells (Sibelius *et al.*, 1996a), and for lipid mediator generation and activation in neutrophils (Sibelius *et al.*, 1999). Infection experiments with isogenic *hly* and *plcA L. monocytogenes* mutants have also suggested that LLO is required for the induction of PlcA-mediated DAG generation and PKC translocation early in macrophage infection (Wadsworth and Goldfine, 2002). CDC-mediated translocation of effector proteins has recently been demonstrated with the related streptococcal toxin SLO, and it has been suggested that this mechanism may play in Gram-positive bacteria a role similar to that of the type III secretion systems in Gram-negative bacteria (Madden *et al.*, 2001; Walev *et al.*, 2001).

LLO may also act as a modulin via a mechanism independent of its membrane-permeabilizing activity. Thus, exposure to non-cytolytic LLO-cholesterol complexes has been shown to induce cytokine gene expression (Nishibori *et al.*, 1996) and IL-1 release (Yoshikawa *et al.*, 1993) in murine macrophages, the release of IL-12, IL-18, and IFN-$\gamma$ in murine spleen cells (Kohda *et al.*, 2002; Nomura *et al.*, 2002; Ito *et al.*, 2003), and the induction of lipid second messengers in human endothelial cells (Sibelius *et al.*, 1996b). Non-cytolytic LLO-cholesterol complexes retain the capacity to bind to cell membranes (see above), and such responses are unlikely to be generated unless the toxin interacts with cell surface receptors, suggesting that CDCs are able to recognize membrane components other than cholesterol. Indeed, it has recently been shown that the human cell-specific CDC Ily from *S. intermedius* uses CD59 as its cellular receptor (Giddings *et al.*, 2004). These observations raise the interesting possibility that LLO released extracellularly and rendered non-cytolytic by complexation with free cholesterol present in body fluids may be targeted to host cell surfaces and mediate signaling events upon binding to specific membrane receptors.

Infection experiments in cell culture models using wild-type and *hly*-mutant *L. monocytogenes* bacteria suggest that intracellularly delivered LLO may also induce signaling events leading to a variety of cell responses. These include activation of the Raf-Mek-MAP kinase pathway in epithelial cells (Tang *et al.*, 1994, 1996; Weiglein *et al.*, 1997), the induction of mucus exocytosis in intestinal cells (Coconnier *et al.*, 1998), cytokine gene expression in macrophages (Kuhn and Goebel, 1994; Nishibori *et al.*, 1996), degranulation and leukotriene formation in neutrophils (Sibelius *et al.*, 1999), and Fas ligand expression on T lymphocytes (Zenewicz *et al.*, 2004). In endothelial cells—a major target cell in the neutrophagogenesis and placental invasion by *L. monocytogenes*—LLO provokes lipid mediator generation (phosphoinositides, DAG and ceramide) (Sibelius *et al.*, 1996a), the up-regulation of adhesion molecules (Drevets, 1998; Kayal *et al.*, 1999, 2002), IL-1 release (Yoshikawa *et al.*, 1996), and nitric oxide (NO) synthesis, and the release of proinflammatory cytokines such as IL-6, IL-8, monocyte chemotactic protein-1, and granulocyte-macrophage colony-stimulating factor (Kayal *et al.*, 1999; Rose *et al.*, 2001). This is associated with the induction of the NF-$\kappa$B signaling pathway via activation of the IkB kinase subunit of the IKK complex, an effect that has been shown to be mediated by LLO in experiments involving heterologous expression of the *hly* gene in the non-pathogenic species *L. innocua* (Kayal *et al.*, 1999, 2002).

Further evidence that LLO is important in modulating the immune response comes from experiments using purified LLO (Carrero *et al.*, 2004a), which showed that the toxin was responsible for lymphocyte apoptosis as typically seen in mouse splenic cells both *ex vivo*, especially in type I IFN-activated lymphocytes (Carrero *et al.*, 2004b), and *in vivo* (Merrick *et al.*, 1997), upon infection with *L. monocytogenes*. The massive regression of the spleen white pulp appears to be pathognomonic of *Listeria* infection in mice (Marco *et al.*, 1991; Conlan, 1996) and may well be caused by LLO-mediated apoptotic lymphocyte depletion. Thus, LLO appears to play dual immunomodulatory roles with similar outcomes in host-parasite interaction, inducing the mobilization of innate host defenses (thereby favoring the spread of infection by making available more host cells for bacterial replication) and at the same time attenuating the T cell-mediated responses it inevitably triggers through its role as
immunodominant, cytosolic-released antigen. This immunosuppressive effect is consistent with other observations linking LLO to the down-regulation of major histocompatibility complex I (antigen I-Ab) and II (antigen H-2Kd) gene expression, as observed in infected murine macrophages (Schüller et al., 1998), and the interference with antigen presentation through the induction of apoptosis in dendritic cells (Guzmán et al., 1996) and type I IFN-sensitized macrophages (Stockinger et al., 2002).

A note of caution must be introduced, however, as although in most cases the above signaling effects can be reproduced by exposure to exogenous purified LLO, it remains unclear whether such effects are directly exerted by the toxin, are due to other bacterial components (which may also be present as contaminants in the toxin preparations), or are just indirect, nonspecific consequences of membrane destabilization or bacterial intrusion into the cytosol.

Finally, a recent study has revealed a facet of LLO that may be highly relevant to pathogenesis: lipid raft clustering. Gekara and Weiss (2004) have shown that the treatment of J774 murine macrophages with LLO led to raft aggregation and clustering of CD14 and CD24 receptors, suggesting that this may be a key mechanism by which the listerial toxin (and potentially any other CDC) modulates host cell responses.

APPLICATIONS OF LLO RESEARCH

With its optimum activity at pH 5.6, its reduced activity at neutral pH, and its short cytosolic half-life, LLO is an ideal molecule for selective perforation of the phagosomal membrane without collateral damage to the host cell. This unique property of LLO has been exploited to develop strategies to introduce therapeutic macromolecules into the cytosol. Needless to say, the discovery of its key role as the immunodominant, cytosolic-released antigen is necessary for the clearance of intracellular pathogens and tumors, via heterologous expression of the listerial hly gene alone or in combination with recombinant antigens. Examples include Escherichia coli (Hu et al., 2004; Radford et al., 2003), Mycobacterium bovis BCG (Hess et al., 1998), or avirulent mutants of Salmonella enterica serovar Typhimurium (Hess et al., 2000) and Bacillus anthracis (Sirard et al., 1997), all of which have provided ample proof of the principle that LLO can indeed mediate the efficient presentation of antigens via the cytosolic MHC class I-restricted pathway (Dietrich et al., 2001).

The considerable amount of knowledge gathered on the pathogenesis of Listeria infection and its molecular determinants make L. monocytogenes bacteria themselves an ideal platform for the rational development of recombinant oral vaccines based on LLO-mediated induction of cellular immunity (Iknomidis et al., 1995; Guzman et al., 1997; Weiskirch and Paterson, 1997; Dietrich et al., 2003). A Listeria-based antigen carrier system has the clear advantage that its gene regulation machinery has naturally evolved to ensure the correct spatio-temporal synthesis of LLO (Kreft and Vázquez-Boland, 2001). Being a Gram-positive bacterium, L. monocytogenes is also devoid of lipopolysaccharide-associated toxicity. Moreover, in addition to CD8+ CTLs, L. monocytogenes also elicits a vigorous Th1-
biaised CD4+ T-cell response (Guzman et al., 1998), possibly because a portion of the bacteria remain entrapped within phagosomes and, consequently, their antigens are processed through the endosomal/phagolysosomal pathway (Figure 40.7). Combined deletions in known virulence genes, such as the actA-plcB operon required for cell-to-cell spread, and in metabolic genes leading to auxotrophy—e.g., genes of the D-alanine biosynthetic pathway involved in the synthesis of the cell wall—can be used to create hyper-attenuated, environmentally safe Listeria vaccine carriers (Thompson et al., 1998; Angelakopoulos et al., 2002). Robust cell-mediated immunity has been achieved using recombinant L. monocytogenes as a vehicle for expression of genes encoding a variety of proteins, including viral (Ikonomidis et al., 1994, 1997; Frankel et al., 1995; Shen et al., 1995) and tumor (Pan et al., 1995; Jensen et al., 1997; Paglia et al., 1997) antigens. Promising results have been obtained in mice with Listeria-based anti-HIV (Mata et al., 1999, 2001; Lieberman and Frankel, 2003) and anti-cancer (Pan et al., 1995; Gunn et al., 2001) vaccines. Protection and even the regression of established tumors have been achieved with the latter, demonstrating the therapeutic potential of this bacterial carrier and the LLO-mediated antigen delivery system for curing neoplastic diseases.

L. monocytogenes can also be used as a vector for transferring DNA into eukaryotic cells. A plasmid inserted with model antigen genes under the control of a eukaryotic promoter and a Listeria phage endolysin gene under the control of the strictly PrfA-dependent actA gene promoter (which is selectively activated in the cytosol) were introduced into an L. monocytogenes strain attenuated by a deletion in the actA-plcB operon. Bacteria underwent lysis upon entry into the cytosol and generated an MHC class I-restricted immune response against the plasmid-encoded model antigens (Dietrich et al., 1998). These experiments demonstrated that Listeria also provides a system of potential value for the development of DNA vaccines and gene therapy tools (Spreng et al., 2000). DNA transfer to host cells has been achieved using LLO-expressing E. coli strains rendered invasive by introduction of the Yersinia pseudotuberculosis inv gene (Grillot-Courvalin et al., 1998).

Other tools for the cytosolic delivery of macromolecules

Alternative strategies exploiting the capacity of LLO to deliver macromolecules to the cytosolic pathway include the co-administration of the toxin (Darji et al., 1995) or its co-encapsulation into pH-sensitive liposomes (Lee et al., 1996; Mandal et al., 2003). Efficient MHC class I antigen presentation and CD8+ responses have been achieved with these methods (Darji et al., 1997; Tanabe et al., 1999; Mandal and Lee, 2003), indicating that they are effective in rerouting the passenger or cargo proteins from the endosomal to the cytosolic antigen presentation pathways. LLO-liposomes (called “listeriosomes”) can be used for bypassing the endosomal/lysosomal-mediated degradation and/or excretion pathway, thus ensuring that encapsulated chemotherapeutic drugs reach their intracellular targets, as recently shown with the plant-derived, antitumoral toxin gelonin (Provoda et al., 2003). Antisense oligonucleotides have also been successfully delivered into the cytosol using listeriosomes (Mathew et al., 2003).

**In vivo expression technology (IVET) systems**

Based on the fact that hly mutants are non-hemolytic on blood agar plates and are significantly impaired in virulence, LLO has been used as both a reporter and a selection system to identify *in vivo*-expressed genes in *L. monocytogenes*. The system is based on the screening of *L. monocytogenes* chromosomal libraries generated by inserting random DNA fragments upstream of a promoterless *hly* gene cloned into a shuttle vector. These libraries are expressed in an *Abhly* mutant, and selection is carried out in mice. Clones with increased survival are isolated, and those carrying constitutive or *in vivo*-induced promoters are differentiated via their respective LLO+ and LLO− phenotypes. Among the *in vivo*-expressed genes identified was *plcA* (Gahan and Hill, 2000; Dubail et al., 2000), a known virulence determinant involved in intracellular survival, providing evidence of the workability of the LLO-based *in vivo* selection system.

**Diagnostic tools**

LLO elicits a potent humoral response during infection (Grenningloh et al., 1998), and several studies have shown that the detection of LLO-specific antibodies can be used in the serodiagnosis of listeriosis and in serological surveys of *Listeria* infection both in humans (Berche et al., 1990; Gholidazeh et al., 1996, 1997) and animals (Miettinen and Husus, 1991; Low and Donachie, 1991; Low et al., 1992; L’hôpital et al., 1993). The *hly* gene has been also used as target for the specific detection and quantification of *L. monocytogenes* by molecular (PCR-based) methods (Nogva et al., 2000; Rodríguez-Lázaro et al., 2004).
Although we have endeavored to cite all major contributions to current knowledge on LLO, due to space limitations or involuntary omission, some may not have been appropriately cited, for which we apologize to the authors. Our research on Listeria is funded by grants from The Wellcome Trust, the Spanish Ministry for Education and Science, and the European Union.

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III. TOXINS ACTING ON THE SURFACE OF TARGET CELLS (EXCEPT SUPERANTIGENS)