Ⅳ. 研究成果の刊行物・別刷

15 Anti-Infective Vaccine Strategies

Toshi Nagata and Yukio Koide

CONTENTS

15.1 INTRODUCTION

Listeria monocytogenes is a Gram-positive, opportunistic intracellular bacterium that can cause serious diseases (e.g., meningitis and death) in immunocompromised hosts, pregnant women, and neonates.[1-3] During the course of its infection, *L. monocytogenes* employs a collection of purposefully made molecules that facilitate its efficient adhesion and entry to host cells, escape from vacuoles, replication in the cytoplasm, and spread to neighboring cells—provoking a cascade of innate and adaptive immune reactions in its wake without being completely eliminated by the host. The host responses to *L. monocytogenes* have been investigated extensively during the past 50 years and have contributed to the understanding of the key concepts in innate and adaptive immunity. Indeed, the *L. monocytogenes* infection model is considered indisputably a paradigm for the study of cell-mediated immunity[4-6] (see chapter 14).

As a food-borne pathogen, the natural route of infection with *L. monocytogenes* is oral ingestion. After crossing the mucosal membrane (i.e., intestine), *L. monocytogenes* eventually settles in a host's dendritic cells and macrophages. Upon escape from the phagolysosome of the host cells by virtue of listeriolysin O (LLO), it replicates rapidly in the cytoplasm, where *L. monocytogenes* antigens are delivered to related immune cells via the major histocompatibility complex (MHC) class I presentation pathway as well as the MHC class II presentation pathway of host cells. Both $CD8^+$ cytotoxic T-lymphocytes (CTL) and type 1 $CD4^+$ helper T-lymphocytes (Th1) have been shown to be amplified during *L. monocytogenes* infection (see chapter 14), and these lymphocytes play a pivotal

role in a host's buildup of protective immunity, as confirmed by experimental depletion and adoptive transfer studies[7–9] or by analysis of mutant mice with a defect in a gene for β2-microglobulin or H2-Aβ.[10,11] Several T cell epitopes (MHC-binding antigenic peptides) in *L. monocytogenes* antigens have been reported.[6,12]

While prior exposures to *L. monocytogenes* virulent strains contribute to the development of an enduring protective immunity in the host against listeriosis, it is not practical to apply intact virulent strains as a vaccine due to obvious health considerations. Hence, a variety of approaches has been taken to attenuate *L. monocytogenes* virulent strains in order for them to be useful in vaccine applications. These include chemical/physical inactivation (or killing), live attenuation, and identification of protective subunits from the bacterium. Although immunization with heat-killed *L. monocytogenes* is in general not sufficiently protective, a combined injection of killed bacteria and LLO leads to a protective immunity against listeriosis.[13] Attenuation of *L. monocytogenes* virulent strains by deletion or replacement of certain virulence-associated genes such as *actA, hly,* and *inlB* or other essential genes has underscored the development of viable, safe, and effective vaccines that mimic natural infection without causing unintended pathogenic consequences. Given that *L. monocytogenes* comprises strains of negligible virulence within the species (e.g., serotype 4a strains), the potential of using these naturally nonpathogenic strains as candidate antilisterial vaccines can be also explored.

Furthermore, vaccine preparations containing defined and subcellular components (e.g., LLO) have been experimented with for protection against listeriosis. Besides its value as a vaccine candidate, attenuated live *L. monocytogenes* has also proven useful as an excellent antigen carrier that delivers target molecules into intracellular environs to strengthen a host's $CD8^+$ and $CD4^+$ T cell responses as well as innate immune responses against other infective agents (e.g., virus and parasite) and cancers. Indeed, compared with other bacterial vectors (e.g., *Mycobacterium bovis* Bacille de Calmette et Guérin (or BCG), *Salmonella, Shigella,* and *Escherichia coli*), *L. monocytogenes* is not only easier to maintain due to its tolerance of extreme stresses, but also has a much higher safety threshold than other bacterial vectors (with 10^{11} organisms being the lethal dose for *L. monocytogenes* wild-type strain vs. 20 organisms for *Salmonella*). Thus, *L. monocytogenes* has increasingly been recognized as a preferred vehicle for delivering protective molecules from various bacterial, viral, and parasitic pathogens.

In this chapter, attenuation of *L. monocytogenes* and the antigen delivery systems based on this bacterium for vaccine development are reviewed. The trials to induce protective immunity against listeriosis with various killed and live *L. monocytogenes* preparations are also examined. Next, the application of attenuated live *L. monocytogenes* for delivering vaccine molecules from other intracellularly localized pathogens including viruses, bacteria, and parasites—protection against which requires involvement of specific cell-mediated immunity—is summarized. Finally, perspectives on the future research that may lead to further improvement in the efficiency and applicability of *L. monocytogenes*-based vaccine strategies against various infective agents are discussed.

15.2 ATTENUATION OF *LISTERIA* FOR VACCINE PURPOSES

Microbial pathogens may lose part or all of their pathogenicity after going through various chemical, physical, biological, and/or genetic treatments. The resulting bacterial preparations can then be applied for protection of hosts against subsequent infections with related bacteria. In addition, identification of defined, protective subcellular components offers another avenue for vaccine development.

15.2.1 Killed Vaccines

Many chemical (e.g., formalin, ether or beta-propiolactone) and/or physical (e.g., heat, ultraviolet, or γ-irradiation) measures are lethal to microbial organisms, and can lead to the killing or inactivation of the bacteria concerned. While these processes may destroy certain antigenic structures and

formations in some bacteria, which are essential for introduction of protective immunity, they seem to have minimal influence on others. This is reflected by the extraordinary success with killed vaccine preparations for prevention of several high-profile bacterial infections in humans (e.g., typhoid fever, cholera, plague, lyme disease, and pertussis). In general, production of the killed or inactivated vaccines is technically simple, with some well-defined and quantifiable manufacturing processes. However, care should be taken to ensure that bacterial aggregates are completely inactivated to prevent some unwanted clinical complication or disease transmission. Additionally, since the killed or inactivated vaccines do not replicate inside the host, they often require multiple injections of relatively high doses to be effective, or occasionally they rely on adjuvants to exert their full immunogenic potential.

The attempts to use killed *L. monocytogenes* preparations for protecting mice against listeriosis have been well documented.[14–17] It is apparent that some of the key antigenic components in *L. monocytogenes* are prone to disruption by chemical or physical treatments, as application of formalin- or heat-killed *L. monocytogenes* has often resulted in minimal immunity against listeriosis in experimental animal models. In Mackaness's landmark paper,[18] in which the importance of cell-mediated immunity for protection against *L. monocytogenes* was highlighted, inactivated *Listeria* failed to protect mice against lethal *Listeria* challenge, to influence the growth curve of *Listeria* in the spleen, and to induce delayed-type hypersensitivity. Many subsequent investigations have since confirmed that heat-killed *L. monocytogenes* (HKLM) does not confer efficient protection[19–23] (see Table 15.1), although some early reports found that HKLM can elicit protective immune responses with the help of polyanions[24] or in C3H/HeJ mouse strain.[25] In the latter case, ineffective killing of *Listeria* at 56°C was suspected for stimulation of such an immunity. It appears that a host's immunity against listeriosis is mediated by lymphoid cells, not by antibodies (Abs),[15,26–30] and that induction of protective immunity by HKLM requires additional manipulations to the host, such as supplemental LLO[13] or IL-12[31,32] injections, anti-CD40 monoclonal antibody (mAb) treatment,[33] or anti-CD4 mAb treatment.[34]

For example, depletion of regulatory $CD4^+$ T cells with anti-CD4 mAb during boost immunization with HKLM dramatically increased the number of *L. monocytogenes*-specific $CD8^+$ T cells, which in turn expressed effector functions such as IFN-γ production.[34] While administration of HKLM alone generally stimulated a T cell response that was insufficiently protective, combining HKLM with purified LLO offered protection to mice against *L. monocytogenes* challenge.[13] Similarly, injection of HKLM together with IL-12 elicited vigorous Th1-type T cell responses and cytokine profiles in mice, resulting in a durable immunity against listeriosis.[31,32] These results suggest that LLO is liable to heat or formalin destruction, and that LLO is an essential component for stimulating production of endogenous IFN-γ at the early stage of immunization and for activation of protective $CD8^+$ T cell against *L. monocytogenes in vivo*. The role of LLO in the generation of endogenous IFN-γ can be overtaken or compensated by IL-12, which induces alternative IFN-γ and IL-2 production through activation of $CD4^+$ Th1 cells, leading to activation of $CD8^+$ CTL. The regulatory $CD4^+$ T cells appear to have a negative impact on $CD8^+$ T cells, as removal of the $CD4^+$ T cells during immunization leads to the expansion of *L. monocytogenes*-specific $CD8^+$ T cells.

On the other hand, Szalay and colleagues[35] reported recently that the protective antigens of HKLM can be introduced into the MHC class I pathway through cross-presentation, facilitating their recognition by host $CD8^+$ T cells and leading to the introduction of a protective immunity. The difference between the early results by others and the study of Szalay and colleagues[35] may be due to the mode of application. In the early reports, injection of HKLM was done only once and not repeatedly as described by Szalay and colleagues,[35] who injected HKLM three times at 5-day intervals. After an examination of immune responses with HKLM immunization, Lauvau and colleagues[36] showed that HKLM immunization primed memory $CD8^+$ T cell populations, which were ineffective at providing protection from subsequent virulent *Listeria* challenge despite their substantial size. This can be attributable to a qualitative difference of memory $CD8^+$ T cells between live *Listeria* infection and HKLM immunization. In addition, HKLM immunization primed $CD8^+$

TABLE 15.1
L. monocytogenes Vaccines against Virulent Strain Challenge

Strain	Attenuation	Mouse Strain	Immunization Route[a]	Immune Responses	Protection	Ref.
		Killed *L. monocytogenes*				
10403	Formalin treatment	Swiss–Webster	i.v.	ND[b]	–	19
Not described	Heat-killed + dextran sulphate (DS 500)	BALB/c	i.p.	ND	+	24
Not described	Heat-killed	C3H/HeJ, C3HeB/FeJ, BALB/c	i.v.	CD8	+	25
Serotype 4b	Heat-killed	C3H/HeJ	i.v.	ND	–	20
EGD	Heat-killed	C57BL/6	i.v., s.c.	CD4	–	21
EGD	Heat-killed	C3H/He	i.v.	CD4	–	22
EGD	Heat-killed	C3H/He	i.v.	CD4	–	23
EGD	Heat-killed	C57BL/6	i.v.	CD8	+	35
ATCC 43251	Heat-killed + IL-12	C3HeB/FeJ, C3H/HeJ	i.p.	CD4	+	31, 32
EGD	Heat-killed + LLO	C3H/He	i.v.	CD4	+	13
EGD	Heat-killed + anti-CD40 mab	C57BL/6	i.v.	CD4, CD8	+	33
EGD	Heat-killed + anti-CD4 mab	BALB/c	i.v.	CD8	+	34
DP-L4056	Δ*uvrAB*	BALB/c, C57BL/6	i.v.	CD8	+	72
10403S	γ-irradiation	C57BL/6	i.v.	CD8	+	38
		Auxotroph Strains				
1070138	Δ*pheA*	MF1	i.v.	ND	+	41
10403S	Δ*dal*, Δ*dat*	BALB/c	i.v.	CD8	+	42
EGD	Δ*aroA*, Δ*aroB*, Δ*aroA/B*, Δ*aroE*	BALB/c	i.v.	CD8	+	46
10403S	Δ*dal*, Δ*dat*, SPAC/pL*idal*	BALB/c	i.v.	CD8	+	44
10403S	Δ*dal*, Δ*dat*, pRRR	BALB/c	i.v.	CD8	+	45
		Virulence Gene-Deficient Strains				
EGD	Δ*hly*	BALB/c	i.v.	CD4	–	56
10403S	Δ*hly*	BALB/c	i.v.	ND	–	57
10403S	Δ*hly*	BALB/c	i.v.	CD8	+	58
10403S	Δ*hly*, CytoLLO	BALB/c, C57BL/6	i.v.	CD8	+	60
LO28	Δ*actA*	C3H	i.v.	CD8	+	62
10403S	Δ*actA*	BALB/c	oral	IgA	ND	64
LO28	Δ*actA*Δ*plcB*, Δ*plcA*	BALB/c	i.p.	CD4	ND	65
10403S	Δ*actA*Δ*plcB*	Human volunteers[c]	oral	IgG, T cells	ND	66
EGD	Δ*actA*, Δ*actA*Δ*plcB*	C57BL/6	i.v.	CD8	+	67
EGD	Δ*actA*, Δ*actA*Δ*plcB*	BALB/c	i.v.	CD8	+	68
EGD	Δ*mpl2*	BALB/c	i.p., oral	CD8, Ab	ND	69

[a] i.p.: intraperitoneal; i.v.: intravenous; s.c.: subcutaneous.

[b] ND: not determined.

[c] This study was carried out in human volunteers, not in mice.

T cell populations that did not acquire effector functions. Upon further investigation on the difference of $CD8^+$ T cell responses between HKLM and live *L. monocytogenes* infection, it is clear that HKLM immunization did not induce as high levels of CD40, CD80, and CD86 expression on $CD11c^{high}$ dendritic cells as live *L. monocytogenes* infection, leading to the difference of $CD8^+$ T cell priming between them.[37]

Datta and colleagues[38] evaluated γ-irradiation as a means to prepare *Listeria* vaccine. Previously, radiation-attenuated *Plasmodium* sporozoite vaccines have been applied against malaria parasites in mice and humans, and protection of humans by immunization with these vaccines has been documented.[39] Whereas the irradiated malaria vaccines were attenuated "live" vaccines, the γ-irradiated *Listeria* vaccine of Datta and colleagues[38] was "killed" *Listeria*. This irradiated *L. monocytogenes* efficiently activated dendritic cells via Toll-like receptors (TLRs) and stimulated protective T cell responses in mice. Immunization with the irradiated *Listeria* strain facilitated cross-presentation of listerial antigens to $CD8^+$ T cells in TAP- and proteasome-dependent manners. Thus, it is possible that γ-irradiation did not alter antigenic and adjuvant structures, which can be destroyed by traditional heat or chemical inactivation. These results indicate that γ-irradiation is applicable to bacterial vaccine candidates including *L. monocytogenes* to induce cell-mediated as well as humoral immunity.

15.2.2 Attenuated Live Vaccine

Bacteria undergoing repeated *in vitro* cultures or passages through non-natural hosts may demonstrate lowered pathogenicity, but their capability to mimic the natural infection and their desired immunogenicity are unaltered. Given that these live attenuated bacteria maintain some self-limiting capacity to infect and replicate in the hosts, show negligible pathogenicity, and yet are highly immunogenic, they have the potential to confer protection against respective infections at relative low doses in the absence of adjuvants. The usefulness of the live attenuated bacteria as vaccines against related bacterial infections is exemplified by attenuated *M. bovis* Bacillus de Calmette et Guérin (i.e., BCG) and *Salmonella typhi* TY21a against human tuberculosis and typhoid fever, respectively. However, a well-known attenuated *L. monocytogenes* strain, ATCC 15313, whose attenuation resulted from successive laboratory subculturing, does not offer protection against listeriosis. The inability of ATCC 15313 to elicit a protective immunity is most likely due to a mutation in its *hly* gene leading to a truncated LLO protein (GenBank Accession No. AY750900), since LLO plays a vital part in *L. monocytogenes'* escape from vacuoles and subsequent stimulation of endogenous cytokine production.[4–6,40] More recently, genetic manipulation techniques have been used to create attenuated live vaccines that lack certain essential genes or key virulence-associated genes, which are the focus of the discussion that follows.

15.2.2.1 Attenuation Strategies

Auxotroph strains. An ideal vaccine strain of *L. monocytogenes* would be absolutely avirulent but fully immunogenic. One well-known strategy to make attenuated bacterial strain is to develop auxotroph strains—namely, strains that require specific nutrients for growth (Table 15.1). Alexander and colleagues[41] described a transposon-insertion mutant of *L. monocytogenes* deficient in prephenate dehydratase gene (Δ*pheA*). The enzyme catalyzes the conversion of prephenic acid to phenylpyruvic acid, which is involved in the biosynthesis pathway of phenylalanine. Mice vaccinated with this mutant *L. monocytogenes* were protected significantly from subsequent challenge with virulent *L. monocytogenes*. However, the mutant persisted in the tissues of infected mice and elicited splenomegaly.

D-alanine is required for the synthesis of the mucopeptide component of the cell walls of virtually all bacteria and is found almost exclusively in the microbial world. Thompson and colleagues[42] constructed an attenuated *Listeria* strain deficient in two genes—that is, an alanine racemase gene

(*dal*), which catalyzes the reaction L-alanine to D-alanine or vice versa, and a D-amino acid aminotransferase gene (*dat*), which catalyzes the reaction D-glutamic acid + pyruvate to α-ketoglutaric acid + D-alanine, or vice versa. The mutant strain can be grown in the laboratory with D-alanine supplement, but is unable to grow without such supplement. Immunization with the strain induced $CD8^+$ T cell responses in mice and generated a protective immunity against lethal challenge by wild-type *L. monocytogenes*, which equaled that elicited by infection with a sublethal dose of wild-type *L. monocytogenes*.

The D-alanine-deficient *Listeria* has also been examined for plasmid DNA delivery.[43] The D-alanine-deficient *L. monocytogenes* mutant (Δ*dal*Δ*dat*) was transformed with a plasmid encoding green fluorescent protein (GFP) under control of the cytomegalovirus immediate early (CMV-IE) promoter/enhancer. No GFP-positive cells in the infected cells were observed when D-alanine was omitted. On the other hand, if D-alanine was supplied at the high concentration or for a lengthy period to allow for sustained bacterial growth, the infected host cells were often killed by the bacteria, resulting in fewer GFP-expressing cells. These results suggest that efficient DNA delivery by transformed *Listeria* must balance invasion and survival of *Listeria* with health and survival of target host cells.[43]

By developing additional systems that obviate the need for exogenous D-alanine administration, the utility of a mutant *Listeria* strain as a vaccine vector can be further improved. Li and colleagues[44] developed a recombinant *Listeria* strain that expresses a copy of the *Bacillus subtilis* racemase gene (*dal*) under the control of a tightly regulated isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter in a multicopy plasmid. The recombinant *L. monocytogenes* demonstrated the strict dose-dependent growth in the presence of IPTG. After removal of IPTG, bacterial growth ceased within two replication cycles. Nevertheless, a single immunization evoked a state of long-lasting protective immunity against wild-type *L. monocytogenes*.

Zhao and colleagues[45] devised a new version of the Δ*dal*Δ*dat* mutant *Listeria* strain, which expresses a *dal* gene and synthesizes D-alanine under highly selective conditions. The suicide plasmid designated pRRR carries a *dal* gene surrounded by two *resI* sites and a resolvase gene, *tnpR*, which acts at the *resI* sites. The resolvase gene is regulated by a promoter activated upon exposure to host cell cytoplasm (*actA* promoter). Therefore, the recombinant *Listeria* (*L. monocytogenes* Δ*dal*Δ*dat*/pRRR strain) is able to grow in liquid culture and to infect host cells without D-alanine supplementation. This system allows only transient growth of the recombinant *Listeria* in infected cells and survival in animals for only 2–3 days. Mice immunized with the mutant *Listeria* produced specific effector and memory $CD8^+$ T cells, and they were protected against lethal challenge by wild-type *L. monocytogenes*.

Stritzker and colleagues[46] constructed a series of *L. monocytogenes* mutants with deletions in genes of the common branch of the biosynthesis pathway leading to aromatic compounds. *aroA* (encoding the first enzyme in aromatic amino acid biosynthesis, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase), *aroB* (encoding the second enzyme, 3-dehydroquinate synthase), and *aroE* (encoding 5-enolpyruvylshikimate-3-phosphate synthase) mutants showed greatly reduced growth rates in epithelial cells and in rich culture media. All *aro* mutants displayed significant virulence attenuation in mice. That is, the 50% lethal dose (LD_{50}) in BALB/c mice was increased at least 10^4-fold for the *aroA*, *aroB*, and *aroA/B* mutants and $>10^5$-fold for the *aroE* mutant compared to the parent strain. Mice immunized with *aro* mutant bacteria developed strong T cell responses and full protection against subsequent virulent *L. monocytogenes* challenge.

Strains lacking essential virulence genes. Another approach to obtain attenuated strains of *L. monocytogenes* is to create bacteria that lack essential virulence genes (Table 15.1). Two of the major virulence factors of *L. monocytogenes* are the pore-forming cytolysin, LLO, and the actin-recruiting and -organizing protein, ActA. LLO is a 58-kDa protein, with essential functions in vacuolar lysis and escape into the cytoplasm of host cells[40] and ActA is necessary for *L. monocytogenes* to spread to adjacent host cells.[47–49] Besides acting as a major virulence factor, LLO is also an immunodominant antigen.[50–52] Previous studies indicate that *L. monocytogenes* with a deficiency in

hly gene encoding LLO was highly attenuated (10^5-fold less virulent[40,53–56]) and that immunization with such a strain generally failed to elicit protective immunity.[56,57]

Recently, Hamilton and colleagues[58] reexamined the role of LLO in listerial immunity by using a recombinant *Listeria* strain that is deficient in most of the *hly* structural gene but expresses an additional $CD8^+$ T cell epitope derived from lymphocytic choriomeningitis virus (LCMV). Injection of the *Listeria* mutant-evoked sizable priming of the epitope-specific $CD8^+$ T cells and development of a functional memory cell population. Further, mice immunized with the *Listeria* mutant were resistant against high-dose challenge of virulent *L. monocytogenes* and also against heterologous challenge with LCMV. It is possible that priming with a low dose of *hly*-deficient *Listeria*, which occurred in an environment with a reduced level of IFN-γ, allowed rapid amplification of antigen-specific $CD8^+$ T cells by booster immunization, despite an undetectable primary response. Thus, the generation of protective immunity by the *hly*-deficient *Listeria* strain may represent a useful platform for vaccine delivery. Harty's group also showed that using dendritic cell vaccine for priming naïve $CD8^+$ T cells in an environment of low inflammation (IFN-γ) accelerated the generation of memory $CD8^+$ T cells.[59] These results indicate that immunization protocols, in addition to selection of attenuated *Listeria* strains, are critical for induction of specific protective immunity.

Bouwer and colleagues[60] described a "cytoLLO" *Listeria* strain, DH-L1233, which harbors a deletion in its *hly* gene (ΔLLO), resulting in a truncated LLO lacking its N-terminal secretion signal sequence (cytoLLO). This strain has an additional mutation in gene encoding PrfA transcriptional activator, leading to constitutive overexpression of the integrated cytoLLO construct and all other PrfA-controlled virulence genes.[61] Although it failed to escape the phagosome and did not replicate within bone marrow-derived macrophages, this strain showed the capacity to deliver native antigens to the MHC class I antigen presentation pathway. Being attenuated 10^5-fold in BALB/c mice compared with wild-type *L. monocytogenes* strain 10403S, it was cleared rapidly in normal and immunocompromised mice. However, antigen-specific $CD8^+$ effector T cells were stimulated after immunization, and mice immunized with the mutant *Listeria* were protected against lethal challenge with a virulent wild-type *L. monocytogenes* strain. In this system, the cytoLLO protein is used for phagosomal lysis after the bacteria die in the phagosome and release antigens into the cytoplasm of host cells, facilitating antigen presentation through the MHC class I pathway.

ActA is an *L. monocytogenes* surface protein involved in the actin nucleation at the bacterial surface and cell-to-cell spread. The *L. monocytogenes actA* mutant could enter and multiply in the cytoplasm of the infected host cell, but had deficiency in actin-dependent cell-to-cell spread. Goossens and Milon[62] showed that the LD_{50} of an attenuated *actA* mutant was 10^3-fold higher than that of the wild-type strain. A single intravenous infection with the maximum sublethal dose of the mutant induced a long-lasting immunity and that protection was mainly conferred by $CD8^+$ T cells, as depletion of $CD4^+$ T cells had no significant effect on the level of protection.[63] Manohar and colleagues[64] applied the *actA* mutant for induction of gut mucosal immune responses. Through oral immunization, the *actA* mutant was capable of colonizing the intestinal mucosa of formerly germ-free mice for a long period without causing disease while eliciting secretory immunoglobulin (Ig) A response. The IgA antibodies recognized the 96-, 60-, 40-, and 14-kDa proteins of *L. monocytogenes*.

Although disruption of the *actA* gene does not interfere with the ability of *Listeria* to replicate in the cytoplasm and subsequent presentation of listerial antigens through the MHC class I pathway, the *actA* deletion may not be sufficient for attenuation of *L. monocytogenes* since these bacteria persist in mice in the liver for up to 7 days.[64] Thus, *L. monocytogenes* mutants containing additional deletions apart from *actA* locus have been examined.[65–67] The *actA/plcB* double mutant was clinically evaluated for safety. In a study involving human volunteers, Angelakopoulos and colleagues[66] showed that 20 healthy volunteers, each receiving a single oral dose (10^6–10^9 CFUs) of *actA/plcB*-deficient *L. monocytogenes* orally, developed no serious long-term health side effects, despite displaying humoral, mucosal, and cellular immune responses to the inoculated *L. monocytogenes* strain. The *actA/plcB* double mutant was also evaluated using the mouse model.[67,68] The double mutant strain was extremely low in virulence and was rapidly eliminated by the murine host

during the first days of infection. Interestingly, the mutant strain exhibited a significantly reduced ability to induce $CD4^+$ T cell-mediated inflammation and a single immunization with the strain efficiently induced and maintained effector memory $CD8^+$ T cells, which protected the immunized mice against wild-type *L. monocytogenes*.

Another virulence gene–deficient attenuated *L. monocytogenes* strain, Δ*mpl2*, which results in the secretion of nonfunctional metalloproteinase (Mpl), has been evaluated as an oral vaccine carrier for a mouse fibrosarcoma.[69] The Mpl proteinase is a zinc-dependent protein, which is required for the production of biologically active phospholipase B for lysis of the double membrane vacuole, and hence for permitting *Listeria* to spread to adjacent cells. Oral and intraperitoneal immunization of mice with this strain stimulated a long-lasting $CD8^+$ T cell response against fibrosarcoma expressing β-galactosidase.

Further, the entire lecithinase operon (consisting of the genes *mpl*, *actA*, and *plcB*)-deficient *L. monocytogenes* strain, designated Δ2, has been constructed[70] and examined as a vaccine carrier.[71,72] Infection of BALB/c mice with the Δ2 strain led to a 10^3–fold higher intravenous LD_{50} value compared with wild-type *L. monocytogenes* EGD strain.[71] Immunization with the Δ2 strain harboring DNA vaccines for *Mycobacterium tuberculosis* Ag85 family antigens has been shown to induce specific T cell responses and protective immunity in mice.[72]

Brockstedt and colleagues[73] reported a metabolically active yet replication-defective *L. monocytogenes* strain that was developed with DNA cross-linking procedure. This *Listeria* strain takes advantage of the potency of live vaccine and the safety of killed vaccine simultaneously as the gene required for nucleotide excision repair, *uvrAB*, is removed, rendering it exquisitely sensitive to photochemical inactivation with psoralen and long-wave-length ultraviolet light. Psoralen treatment of this strain leaves intact the ability of a population of inactivated bacteria to express its genes, but prevents its productive growth and its disease-causing ability in the immunized host. The psoralen-inactivated *L. monocytogenes* Δ*actA*/Δ*uvrAB*-OVA, which has ovalbumin (OVA) gene as a model antigen gene, was capable of inducing OVA-specific $CD8^+$ T cells and protective immunity in mice against OVA-expressing vaccinia virus and also virulent wild-type *L. monocytogenes* challenge.

Besides these virulence factor–deficient mutants, newly identified virulent factors can be also targeted for development of new attenuated *Listeria* strains. A new approach to identify genes involved in the virulency of *Listeria* using signature-tagged transposon mutagenesis has been reported.[74]

Suicide gene. In addition to the aforementioned attenuation strategies for *Listeria*, a "suicide" gene has been used to ease the destruction of the bacterium. A typical suicide gene that has proven useful in the preparation of attenuated *Listeria* vaccines is an endolysin gene, *ply118*, derived from *Listeria* temperate phage A118 attacking *L. monocytogenes* serovars 1/2.[75] This gene encodes for a cell-wall lytic enzyme that cleaves between L-alanine and D-glutamate residues of listerial peptidoglycan, but does not cleave the peptidoglycan of other bacteria in infected host cells.

Antibiotics. Antibiotics treatment (e.g., penicillin, streptomycin, and tetracycline) is very efficient in killing *L. monocytogenes in vivo*.[71,76] Ampicillin administration with the drinking water appears to influence the duration of *L. monocytogenes* infection in mice and on the magnitude of T cell responses[77–79] and has been shown to eliminate viable bacteria from spleens of *L. monocytogenes*-infected mice within 12 hours after administration of the antibiotic.[80] Mice undergoing repeated *L. monocytogenes* infection followed by antibiotic treatment may acquire an increased level of immunity against listeriosis.

15.2.2.2 Pros and Cons of Attenuated Live Vaccines

Schafer and colleagues[81] were the first to demonstrate the feasibility of using *L. monocytogenes* as an efficient attenuated live vaccine for induction of cell-mediated immune responses, especially $CD8^+$ T cell responses. Since then, many live attenuated *L. monocytogenes* vaccines have been developed. These live attenuated vaccines possess some clear advantages and potential drawbacks in comparison with other vaccine strategies, which are discussed next.

The advantages of these vaccines include:

- Propensity to infect antigen-presenting cells (APCs). *L. monocytogenes* specifically infects professional APCs, which include monocytes, macrophages,[82,83] and dendritic cells (DCs).[84,85] After being taken up by these cells, some bacteria escape from the phagocytic vacuoles into the cytoplasm and grow there. *L. monocytogenes* protein antigens are then presented to the MHC class I pathway to $CD8^+$ T cells. In addition, bacterial protein antigens are also delivered to $CD4^+$ T cells via the MHC class II pathway during listeriosis (see chapter 14). Thus, *L. monocytogenes* represents an ideal carrier for antigen-delivery to elicit a cell-mediated immune response.
- Ease of genetic manipulation. *L. monocytogenes* can be transformed relatively easily with plasmid vaccine vectors using electroporation, although the transformation efficiency of *L. monocytogenes* is lower than that of *Escherichia coli*.[86] Methods for growing and processing *L. monocytogenes* have been established,[87,88] along with an increasing number of genetic tools available for its genetic manipulation.[89] For example, shuttle vectors have been developed to facilitate easy insertion of foreign genes stably into the *Listeria* chromosome[90] or to allow conjugative transfer of plasmid DNA from *E. coli* to *L. monocytogenes*[91,92] (see chapter 9).
- Adjuvanticity. Murine immune responses to *L. monocytogenes in vivo* are essentially of Th1-type, in which interferon (IFN)-γ and interleukin (IL)-12 productions are predominant.[93,94] Cell-surface moieties of *L. monocytogenes* are recognized by host cells as "danger" signals, against which innate immune responses are provoked via the TLRs, the pattern recognition receptors on host cells. Cell activation by lipoteichoic acid and soluble peptidoglycan, the main stimulatory components of Gram-positive bacteria, has been shown to be mediated by TLR2.[95] Since lipopolysaccharide (LPS) in the cell wall of Gram-negative bacteria can cause severe side effects such as septic shock *in vivo*, Gram-positive bacteria constitute a safe and preferred option for vaccine delivery.

  Lipoteichoic acid, a predominant surface glycolipid of *L. monocytogenes* potently induces IL-12 p40 gene expression similar to LPS.[96] IL-12 has been shown to augment IFN-γ production by natural killer and T cells. Lipoteichoic acid derived from *L. monocytogenes* has been also shown to induce a transient IκBα degradation. After *L. monocytogenes* enters the cytoplasm of the host cell, its virulence genes, *plcA* and *plcB*, express two phospholipases, which lead to persistent induction of NF-κB DNA-binding activity.[70] In turn, this activity induces expression of a series of target genes including IL-12 p40 gene.

  Furthermore, the unmethylated cytidine–phosphate–guanosine (CpG) sequence abundant in *Listeria* genomic DNA may stimulate innate immunity through TLR9-mediated signal. Therefore, live attenuated *Listeria* acts as a "natural adjuvant," thereby attracting cells involved in innate immunity and promoting APC maturation and activation. Thus, dendritic cells, by far the most potent APCs, are activated to express the necessary costimulatory molecules that enhance subsequent antigen presentation activity, and bias the ensuing immune response in the direction of cell-mediated immunity.
- Simplicity of handling and storage. *L. monocytogenes* is a robust bacterium that can be stored as lyophilized powder, similar to the current procedure for *Mycobacterium bovis* BCG vaccine. In addition, it is easily controlled by common antibiotics such as penicillin and tetracycline, an option that is usually unavailable for viral vectors. In the case of a *Listeria*-carrying DNA vaccine system, plasmids for DNA vaccine do not require purification, which is an indispensable step for naked DNA vaccination.
- Mucosal route of immunization. Many human infectious diseases are initiated at mucosal surfaces. Attenuated live *Listeria*-carrying vaccine can be administered effectively by any one of several mucosal routes (e.g., oral, intranasal, intragastric, intravaginal, or rectal).[69,97] Administration of *L. monocytogenes* vaccines through these routes often elicits the immune

responses in the host that emulate those by natural infections and can lead to long-lasting protective mucosal and systemic immunity. Moreover, vaccination via a mucosal route is associated with fewer side effects and in many cases lower delivery costs. One caveat of the murine model of the oral *Listeria* vaccine system would be that murine E-cadherin, a *Listeria* surface protein that serves as a receptor for internalin A, was reported to have a glutamine to a proline substitution.[98,99] As *L. monocytogenes* surface proteins InlA and InlB are responsible for invasion of nonphagocytic cells and seem to determine the specificity of the cell type to be infected, wild-type murine models of *Listeria* infection are not appropriate for addressing internalin function *in vivo.*

- Amplification of DNA vaccine plasmids *in vivo.* The low efficiency of traditional naked DNA vaccination can be due to the limited amounts of DNA vaccine plasmids available *in vivo.* As *L. monocytogenes* harboring DNA vaccine plasmid allows the plasmid replication inside, the amounts of *Listeria*-carrying DNA vaccines will increase *in vivo.*

Like other attenuated bacterial vaccines and naked DNA vaccines, *Listeria*-carrying vaccines have several biosafety risks. Potential drawbacks include:

- potential risk of integration of *Listeria*-carrying genes to the host cell chromosome
- the spread to, and long-term persistence of, the plasmid in multiple tissues
- the induction of tolerance to the immunization antigens
- the risk of autoimmune disease by elicitation of anti-DNA Abs
- possibility of reversion to toxic/virulent phenotype

A live vaccine strain should contain appropriate attenuation mutations in genes that are essential for survival in the host cells. Strains containing more than two genetically unlinked attenuating mutations are infinitely better than those that have one mutation. However, too much attenuation may reduce immunogenicity of the vaccine. Therefore, the balance between attenuation (safety) and maintaining immunogenicity of vaccines is one of the important issues for clinical adaptation of vaccine candidates.

The bacterial restriction and modification system may sometimes hamper the maintenance of plasmids introduced into *L. monocytogenes.* Host specificity in a bacterial strain is the result of the action of particular enzymes that impose a "modification" pattern on DNA. The pattern identifies the source of the DNA. Modification allows the bacterium to distinguish between its own DNA and any "foreign" DNA, which lacks the characteristic host modification pattern. This difference renders an invading foreign DNA susceptible to attack by restriction enzymes that recognize the absence of methyl groups at the appropriate sites. Such "modification and restriction" systems are widespread in bacteria, although some bacterial strains lack any restriction system. *E. coli* strains widely used in laboratories all over the world have mutations in these systems by genetic manipulation. However, live *Listeria* strains used as vaccine carriers may still have these systems and interfere with maintenance of exogenous plasmids in them after introduction of plasmids by electroporation. These "modification and restriction" systems will not be a problem after introduction of plasmids by conjugation.

Intrinsic immunogenicity of viable bacteria itself is another issue. Live attenuated *L. monocytogenes* is composed of a variety of proteins, lipid, sugar, and so on, which are themselves immunogenic in host cells. So, repeated immunization of live attenuated *Listeria* vaccine is possible to cause rapid elimination of live *Listeria* vaccine. A similar situation has been noticed with adenovirus-based vaccines. One useful strategy to overcome this possibility is to combine different vaccination methods (so-called prime-boost strategy). This is achieved by immunization with naked DNA

vaccination first, and followed by boost immunization with live *Listeria* vaccine.[100–102] Alternatively, it can be done by injection with different carrier bacteria for each immunization step.

Preexisting immunity to *Listeria* is also one of the issues concerning the use of attenuated *Listeria* carrier vaccines. *L. monocytogenes* is ubiquitously distributed in nature, being found commonly in soil, decaying vegetation, water, and as part of the normal flora of mammalian intestines[2] (see chapter 2). However, the incidence of listeriosis is low. Therefore, preexisting immunity to the vector may not be widespread in humans. After evaluating whether existing antilisterial immunity limits or alters efficacy of *Listeria* vaccine, several investigators confirmed that antilisterial immunity does not inhibit the development of recall responses as well as primary immune responses to antigens delivered by the *Listeria* vaccine system and that preexisting immunity to *L. monocytogenes* does not prevent induction of immune responses by *Listeria* vaccines.[103–105]

15.2.3 Naturally Avirulent Live Vaccines

Although *L. monocytogenes* has been regarded as being pathogenic at the species level, it encompasses a diversity of strains with varied pathogenic potential. Of the 11 common serotypes (i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, and 4e), three (1/2a, 1/2b, and 4b) have accounted for over 98% of clinical cases of human listeriosis. Through analysis in mouse models, it is clear that except for serotype 4a, all other *L. monocytogenes* serotypes are potentially virulent and able to cause mortality in the A/J mouse strain via an intraperitoneal route. Given that naturally avirulent serotype 4a strains are not isolated from human patients and are readily eliminated by mammalian hosts, they can be useful as candidate vaccine against virulent strain challenge,[106–109] and also as a vaccine vehicle for delivery of protective molecules against other microbial pathogens.

The concept of using naturally avirulent bacterial strains as vaccines against virulent strains has been explored successfully in several bacterial systems. For instance, an avirulent live *Lawsonia intracellularis* strain administered orally to pigs facilitated the development of protective immunity against challenge with virulent *L. intracellularis*.[110] A *Mycoplasma gallisepticum* (MG) isolate (K5054) causing very mild lesions prevented challenge infection with a virulent MG strain in turkeys.[111] Immunization of chicken with avirulent *Salmonella* carrying *Campylobacter cjaA* gene hindered the heterologous wild-type *C. jejuni* strain to colonize the bird cecum.[112] Furthermore, mice injected with naturally avirulent *L. monocytogenes* serotype 4a strains ATCC 19114, L99, and HCC23 developed a strong immunity (both short and long term) against virulent strains' challenges.[106–109]

Originally isolated from catfish, serotype 4a strain HCC23 is nonpathogenic in mouse virulence assay, and it lacks several virulence-specific genes such as those encoding internalins (*lmo2821* and *lmo2470*) and transcriptional regulators (*lmo0833, lmo1116, lmo1134,* and *lmo2672*). Recent DNA sequencing analysis revealed that, apart from a deletion of 105 nucleotides in its *actA* gene leading to a reduction of 35 amino acids in the encoded ActA protein, HCC23 possesses largely intact *prfA, plcA, hly, mpl,* and *plcB* genes in the *prfA* virulence gene cluster in comparison with EGD (GenBank accession nos. AY878649 and DQ118415). Whereas EGD ActA protein possesses four copies of proline-rich repeats (DFPPPPTDEEL) that control *L. monocytogenes* movement between cells (with each repeat contributing about 2 μm/min), the deletion of 35 amino acids in HCC23 ActA protein effectively removes two copies of the proline-rich repeats (Genbank accession no. DQ118415). Thus, it is no surprise that while serotype 4a strains readily replicate within fibroblast L929 and macrophage J774 cells, they are unable to undergo cell-to-cell spread. Apparently, serotype 4a strains produce a nonfunctional ActA protein that is 5 kDa smaller than that from virulent strain EGD.[106–109]

IFN-γ is a vital component in the cell-mediated immune response, which activates antimicrobial macrophages and NK cells for subsequent development of host natural resistance and adaptive

immunity to listeriosis. In contrast to mice injected with virulent strain EGD that produced a large amount of IFN-γ (about 580 pg/ml of serum on day 2 after exposure), mice injected with avirulent strains HCC23 and ATCC 19114 generated a much lower level of IFN-γ production (about 50 pg/ml of serum on day 2 after exposure). However, similar to mice immunized with virulent strain EGD, mice immunized with avirulent strains HCC23 and ATCC 19114 all developed a strong immunity against *L. monocytogenes* challenge.[108,109] These results suggest that in spite of generating a relatively smaller (or negligible) amount of IFN-γ than virulent strain EGD, avirulent strains HCC23 and ATCC 19114 are equally efficient in initiating a protective immunity against listeriosis. It is thus apparent that induction of excessive amounts of IFN-γ, such as in the case with EGD, may not only be unnecessary, but actually detrimental to the host. As IFN-γ enhances secretion of TNF-α, it may bring such a potent immune response that the host may succumb as a consequence, and naturally avirulent serotype 4a strains may cause much less potential harm to the vaccine recipient through reduced IFN-γ and TNF-α production.

Besides being useful as a candidate vaccine against listeriosis, *L. monocytogenes* avirulent serotype 4a strains also hold promise as a potential vaccine carrier for other bacterial, viral, fungal, and parasitic diseases as well as cancers. The application of a naturally avirulent strain not only negates the necessity to attenuate *L. monocytogenes* virulent strains through deletion or replacement of certain virulence-associated genes such as *actA*, *hly*, and *inlB*, but provides a much safer approach for effective delivery of vaccine molecules. Further study is required to insert a foreign gene into a site (e.g., *actA*) in an avirulent *L. monocytogenes* serotype 4a strain followed by assessment of its vaccine efficacy. This will help verify the potential of using naturally avirulent strains as an effective alternative to live attenuated *L. monocytogenes* strains for vaccine delivery.

15.2.4 Subunit Vaccines

Built on a detailed knowledge of the molecular and immune mechanisms of bacterial pathogens, it becomes possible to utilize more defined, protective subcellular components for enhanced vaccine efficiency and safety. This not only helps eliminate some unquantifiable and unintended side effects associated with the use of whole cell vaccines, but also gives batch-wise reliability and consistency. Similarly, utilizing plasmids, which are replicable in *E. coli* but not in hosts, to express protective proteins *in situ* creates so-called DNA vaccines that further improve the vaccine efficacy and streamline the vaccine production processes. The subcellular bacterial vaccines against diphtheria/tetanus/pertussis (acellular), *Hemophilus influenza* b, *Streptococcus pneumoniae*, and meningococci are some of the excellent examples of the types of vaccines that have been successfully developed.

As the 58-kDa LLO of *L. monocytogenes* is required for the initiation of specific immunity, many innovative designs incorporating this protein have been described to enhance vaccine potency.[113–121] *L. monocytogenes* LLO is a pore-forming, cholesterol-dependent cytolysin belonging to a family of homologous proteins present in several pathogenic bacteria, such as perfringolysin O derived from *Clostridium perfringens* and streptolysin O from *Streptococcus pyogenes*. Using plasmid DNA constructs encoding recombinant forms of LLO, peptide-specific $CD8^+$ immune T cells exhibiting *in vitro* cytotoxic activity were activated in immunized mice, and provided protection against a subsequent *L. monocytogenes* challenge.[113–115] Injection of IFN-γ or IFN-γ receptor knockout BALB/c mice with plasmid DNA constructs encoding recombinant forms of the *L. monocytogenes* LLO or a minigene encoding LLO 91–99 (GYKDGNEYI) elicited the peptide-specific $CD8^+$ T cell response and also resulted in protection against listeriosis.[116,117] A recombinant LLO 91–99 minigene vaccinia virus infection provided partial protection against listeriosis.[118] Injection with LLO 91–99-coated DCs[119] or DCs retrovirally transduced with LLO 91–99 minigene[120] protected mice against high-dose challenge with virulent *L. monocytogenes*. Mice immunized with plasmid DNA constructs for an Ii-LLO 215–226 fusion protein in which LLO 215–226 is a dominant Th epitope also developed significant protective immunity against *L. monocytogenes*.[121]

15.3 *LISTERIA*-BASED ANTIGEN DELIVERY SYSTEMS

15.3.1 DELIVERY OF ANTIGENIC PROTEIN

Listeria-carrying vaccines that produce antigenic proteins of interest have been used to induce cell-mediated immunity, especially specific $CD8^+$ T cell responses.[122–124] Essentially, there are two ways to deliver antigens using the attenuated *Listeria* vaccine system. One is antigenic protein delivery by *Listeria* that itself produces antigens, and the other is to use attenuated *Listeria* as a DNA vaccine carrier (see section 15.3.2). Secreted proteins of intracellular bacteria such as *Listeria* and *Salmonella* have been shown to be good inducers of cell-mediated immune responses because bacterial secreted proteins are vulnerable to the protein degradation system of host cells and the antigen processing and presenting systems. Only secreted (not nonsecreted) proteins of *Salmonella* have been shown to be effectively presented by the MHC class I molecules, followed by induction of $CD8^+$ T cell responses.[125,126] In contrast, infection of mice with recombinant *L. monocytogenes* expressing a model epitope in either secreted or nonsecreted form resulted in similar $CD8^+$ T cell priming efficacy by a cross-presentation mechanism.[127–129] Interestingly, Shen and colleagues[127] reported that antigen-specific $CD8^+$ T cells conferred protection only against *Listeria* expressing the secreted antigen, but not against *Listeria* expressing the nonsecreted form of the same antigen, thus indicating that nonsecreted antigens in *L. monocytogenes* have immunogenicity for $CD8^+$ T cells, but do not necessarily become targets of the $CD8^+$ T cells.

Foreign genes encoding antigens of interest may be carried on plasmid or integrated in the *Listeria* genome. Plasmids harboring foreign genes are maintained episomally in *Listeria*. A number of plasmid vectors developed for use in other Gram-positive bacteria have been applied successfully in *L. monocytogenes* and several of these have been improved or modified. A typical plasmid for antigen production in *Listeria* is shown in Figure 15.1A. The plasmid should contain replication origins of both *L. monocytogenes* and *E. coli*. A replication origin of pAMβ1 derived from *Streptococcus faecalis*[130]

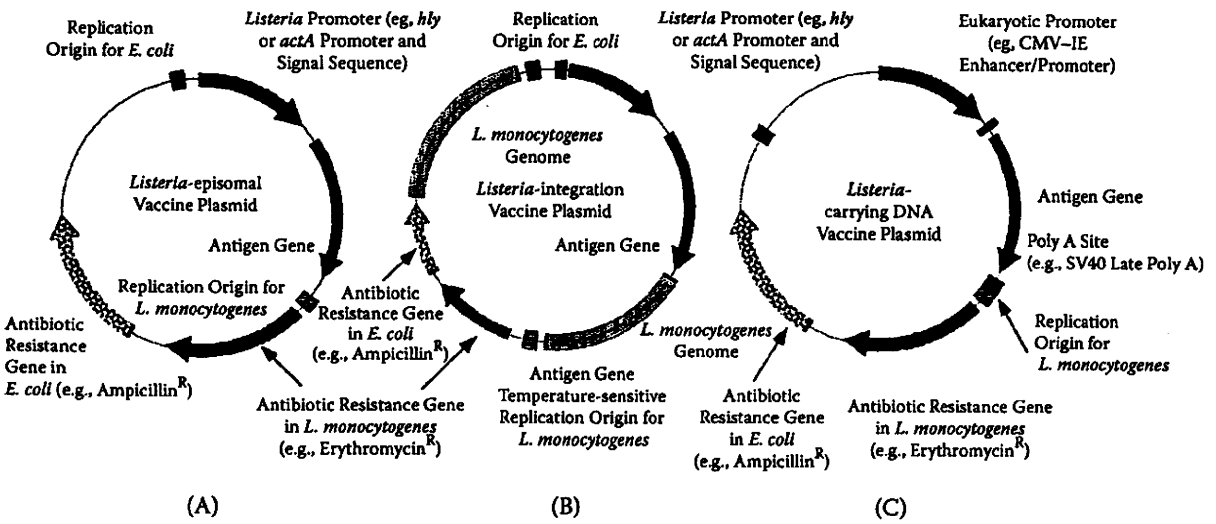

FIGURE 15.1 Schematic drawings of typical plasmids for antigen expression in *Listeria*-carrying vaccine system. (A) A typical plasmid for antigen expression in *Listeria* contains replication origins for *L. monocytogenes* and *E. coli*, antibiotic resistance genes for *L. monocytogenes* and *E. coli*, and a gene that encodes the target antigen, which is driven by a strong *Listeria* promoter. In the case of genome integration plasmid (B), upstream region and downstream region of the antigen gene should be *Listeria* genome sequence for homologous recombination with *Listeria* genome and a temperature-sensitive origin of replication of *Listeria* facilitates integration of the plasmid into *Listeria* genome. (C) A plasmid for antigen expression in infected host cells contains replication origins for *L. monocytogenes* and *E. coli*, antibiotic resistance genes for *L. monocytogenes* and *E. coli*, and a gene that encodes the target antigen, which is driven by a strong eukaryotic promoter such as CMV-IE enhancer/promoter.

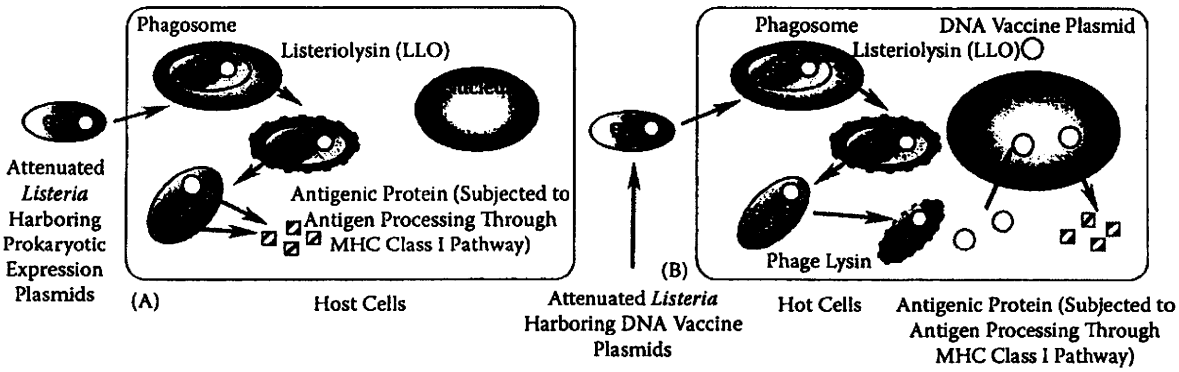

FIGURE 15.2 Schematic drawings of antigen delivery system by *Listeria*-carrying vaccines, with antigens being produced either in *Listeria* (A) or in infected host cells (B). (A) An attenuated *Listeria* strain harboring vaccine plasmid or an integrated antigen gene in the genome infects macrophages and is released into the cytoplasm of infected host cells by virtue of LLO of *L. monocytogenes*. The bacterium produces the antigenic proteins (usually secreted proteins) *in situ* in the cytoplasm of the infected host cells by using *Listeria* transcription and translation machinery. Produced antigenic proteins are then subjected to antigen processing and presentation, mainly through the MHC class I pathway, leading to specific $CD8^+$ T cell responses. (B) An attenuated *Listeria* strain harboring DNA vaccine plasmids infects macrophages and is released into the cytoplasm of infected host cells as in (A). Then, if plasmids contain a suicide gene such as listeriophage gene, *ply118*, the gene product lyses *Listeria* from inside and releases DNA vaccine plasmids into the cytoplasm of the infected host cells, allowing expression of the antigen by using host cell transcription and translation machinery. Produced antigenic proteins are subjected to antigen processing and presentation systems of host cells, mainly through the MHC class I pathway, leading to specific $CD8^+$ T cell responses.

has been the most adequate for stable replication in *L. monocytogenes*.[131] Antibiotic resistance genes in *L. monocytogenes* and also *E. coli* have been employed to facilitate selection of *L. monocytogenes* and *E. coli* possessing the plasmid. A heterologous antigen gene is driven by *Listeria* promoter, usually the *hly* (encoding LLO) or *actA* promoter. Codon usage change may be necessary for high expression of the heterologous antigen gene, as optimal codons for expression of proteins in a variety of organisms substantially differ. For example, *L. monocytogenes* usually used A+T-rich codons and *M. tuberculosis* tends to use G+C-rich codons. Introduction of plasmid DNA into *L. monocytogenes* can be accomplished by transformation of *L. monocytogenes* protoplasts[132] or by electroporation.[86]

The antigen delivery mechanism by *Listeria* is shown in Figure 15.2(A). *L. monocytogenes* is released to the cytoplasm of host cells from phagosomes with assistance from LLO, and antigenic proteins produced in recombinant *L. monocytogenes* are secreted to the cytoplasm of host cells, which are then subjected to antigen processing and presenting machinery of host cells through the MHC class I and also class II pathways. Usage of high-copy-number plasmids would lead to a high yield of foreign antigens in *Listeria*, although the forced expression of foreign genes might be deleterious in terms of growth of the bacteria. One of the issues pertaining to this episomal system is that the plasmid would be cured easily *in vivo* without selection pressure. However, as elimination of *Listeria* just 1 day after infection has been reported to have minimal impact on $CD8^+$ and $CD4^+$ T cell responses, short-time antigen display *in vivo* with recombinant *Listeria* vaccines may be sufficient for induction of immune responses.

Ikonomidis and colleagues[133] reported that infection of mice with the mutant *L. monocytogenes* secreting the LLO-influenza virus nucleoprotein (NP) fusion protein (Lm-NP) led to *in vivo* priming of CTLs in BALB/c mice, which recognized and lysed influenza virus–infected syngeneic target cells. The gene coding for the transcriptional activator prfA was introduced on the plasmid, as PrfA is a key transcription factor for a series of virulent factor genes including LLO and necessary for maintaining virulence of the bacterium. A replication-defective *prfA* (–) *L. monocytogenes* strain was transformed with the plasmid, and the resulting transformants retained the plasmid stably in the episomal state *in vivo* and efficiently secreted the fusion protein.

Verch and colleagues[134] developed a new system in which D-alanine racemase-deficient *Listeria* mutant (Δ*dal*Δ*dat*; *Lmdd*)[42] carrying vaccine plasmids was utilized. The vaccine plasmid incorporated *Listeria dal* gene driven by *Listeria* p60 promoter, whose expression supplements the requirement of exogenous D-alanine for the bacteria growth. Retention of the antibiotic-resistant gene-free plasmid in the *Listeria* carrier in the system resulted in the development of *Listeria* episomal vaccines without using the antibiotic-resistant gene, which complies with Food and Drug Administration regulations.

Alternatively, delivery of antigenic protein by *Listeria* can be achieved through the genome integration system, in which foreign antigen genes are integrated in *Listeria* genome, which allows stable retention of foreign genes. Several different strategies for integration of a heterologous antigen gene into *Listeria* genome have been reported. The plasmids can be grouped according to their usages: plasmids used for the delivery of transposons into *L. monocytogenes*[135] and plasmids used for homologous recombination with *Listeria* chromosomal sequences. Gram-positive, temperature-sensitive origins of replication have been utilized to facilitate integration and excision of plasmid vectors. One representative scheme is shown in Figure 15.1B. In this case, integration of the antigen gene is accomplished after two successive homologous recombination events in two *Listeria* genome homologous regions in the plasmid. The temperature-sensitive replication origin of *L. monocytogenes* facilitates the events as mentioned before. After introduction of the plasmid into *L. monocytogenes* by electroporation, the plasmid-carrying bacteria are selected by antibiotic addition. Then the antibiotic-resistant bacteria are subjected to growth at nonpermitted temperature (e.g., 40°C) to prevent replication of the plasmid. Thus, only bacteria that integrate the plasmid into the genome are positively selected. Once the plasmid is integrated into *Listeria* genome, the system does not require the presence of an antibiotic to select for the retention. It is possible for the expression level to be low due to low copy number (usually one copy) of a foreign gene in *Listeria* genome, but selection of an appropriate promoter for expression of a foreign gene will help to enhance the antigen expression.

Shen and colleagues[136] used the genome integration system for attenuated *Listeria* vaccines against LCMV. Several plasmids for stable, site-specific integration of antigen expression cassettes into the *Listeria* chromosome were constructed by sequential homologous recombination events. The plasmids were designed to drive an antigen gene, LCMV-NP, by the promoter of the *hly* gene and to integrate the antigen gene into the *Listeria* chromosome region just downstream from a transcriptional terminator between the lecithinase (*plcB*) and lactate dehydrogenase (*ldh*) operons. LCMV antigen expression was regulated *in vitro* in a manner identical to the chromosomal *hly* locus and expression of LCMV-NP was enough to induce perfect protection against LCMV challenge infection.

Lauer and colleagues[137] developed a *Listeria* site-specific phage integration vector system (i.e., pPL2 plasmid), which carries listeriophage PSA integrase and PSA *attPP′* sites to integrate a foreign gene into the 3′ end of an arginine tRNA gene of the *L. monocytogenes* genome. As commonly used *L. monocytogenes* strains 10403S and EGDe do not have a prophage at the $tRNA^{Arg}$ attachment site, pPL2 plasmid is readily utilized in these backgrounds for integration-type attenuated *Listeria* vaccine construction. In addition, the pPL2 plasmid can integrate in an *L. monocytogenes* strain that already carries a prophage at the $tRNA^{Arg}$ attachment site, such as LO28, which further increases the usefulness of pPL2 as a genetic tool for integration of antigen genes into the *L. monocytogenes* genome.

15.3.2 Delivery of DNA

Another way to deliver antigens is to use attenuated *Listeria* as a DNA vaccine carrier—in other words, as a vehicle of genes encoding target antigens. This bacteria-mediated DNA delivery to host mammalian cells has been designated as bactofection.[138,139] Bacteria as DNA vaccine carriers for genetic immunization have been reviewed by several investigators.[123,140–151] Usually, the genes are driven by mammalian promoter (Figure 15.1C). *Listeria* is used as only a vehicle/vector to transport

the gene into the eukaryotic cells. The released plasmids get into the nucleus and the transgene is expressed by the eukaryotic transcription and translation machinery (Figure 15.2B). The ability of *L. monocytogenes* to enter the cytoplasm of host cells after phagocytosis and deliver plasmid DNA directly to the cytoplasm makes it an attractive DNA delivery candidate to induce cellular immune responses.

RNA interference has been used for post-transcriptional gene silencing, which has been observed in many eukaryotic cell types. Genetic interference with specific double-stranded RNA has been reported via bactofection.[152] By feeding recombinant *E. coli* to *Caenorhabiditis elegans*, a free-living nematode, it may offer a new approach for construction of attenuated *Listeria* strains for antigenic delivery.

Naked DNA vaccine strategy has a variety of advantages over other vaccine methods,[153,154] but its chief weaknesses include low immunogenicity, a need for adjuvant, and the high cost of purifying injection-grade plasmid DNA. Use of *Listeria* to deliver plasmid DNA negates the need to purify plasmid prior to injection, and thus it provides a more efficient and low-cost alternative to the naked DNA vaccine strategy.

Figure 15.1C illustrates a typical plasmid for a *Listeria*-carrying DNA vaccine system. Structure of plasmid DNA for a *Listeria*-carrying DNA vaccine system is essentially the same as that of the plasmid DNA for naked DNA vaccination. DNA vaccines are composed of an antigen-encoding gene whose expression is driven by a strong eukaryotic promoter such as CMV I.E. enhancer/promoter. The plasmid possesses a polyadenylation termination sequence such as the sequence derived from simian virus 40 or bovine growth hormone gene and a selective marker such as ampicillin resistance gene to facilitate selection of *E. coli* containing the plasmid. Usually, plasmids for naked DNA vaccines include special nucleotide sequences for enhancing the immunogenicity and an unmethylated CpG dinucleotide with appropriate flanking regions. The CpG motif stimulates the innate immune system through TLR9 to produce a series of immunomodulatory cytokines such as IL-12 and IFN-γ, which promote the development of Th1 cells.[155–157] However, in the case of *Listeria*-carrying DNA vaccines, components of *Listeria* itself, such as lipoteichoic acids, stimulate the innate immune system. Therefore, the contribution of the CpG sequence in *Listeria*-carrying DNA vaccines as an immune adjuvant is low. Plasmid DNA for *Listeria*-carrying DNA vaccines should contain an origin of replication for *Listeria*. Introduction of plasmid DNA into *L. monocytogenes* is again accomplished by transformation of *L. monocytogenes* protoplasts[132] or by electroporation.[86]

Hense and colleagues[76] evaluated *Listeria* as a vehicle for gene transfer using a variety of cell lines. Gene transfer to host cells was achieved after treating host cells infected with plasmid-carrying *Listeria* with tetracycline, an antibiotic that is only bacteriostatic, as tetracycline treatment makes the bacteria susceptible to cellular defense and degradation mechanisms. It appeared that bacterial properties required for delivery of the eukaryotic expression plasmids were strictly dependent on the ability of *Listeria* to both invade eukaryotic cells and egress from the vacuole into the cytoplasm of the infected host cells, and that macrophage-like cells or primary, peritoneal macrophages were almost refractory to *Listeria*-mediated gene transfer.

Dietrich and colleagues[71] demonstrated the feasibility of a DNA vaccination system of an attenuated self-destructing *L. monocytogenes* strain in a cell culture system. A deletion mutant strain of *L. monocytogenes*, Δ2, that lacks the entire lecithinase operon, including the virulence-associated genes *actA*, *mpl*, and *plcB*, can infect macrophages and replicate in the cytoplasm of host cells, but cannot spread to adjacent cells.[70,71] This attenuated mutant was introduced with a plasmid containing the gene for lysis protein PLY118 of the listerial bacteriophage A118. The *ply118* gene expression is controlled by the *actA* promoter, which is active when *L. monocytogenes* is in the host cell cytoplasm. Thus, this *L. monocytogenes* mutant escapes from the phagosome and then lyses when the *ply118* gene is expressed in the cytoplasm of host cells. Autolysis of the *L. monocytogenes* mutant apparently releases the plasmid DNA into the host cell cytoplasm, allowing expression of the transgene in the host cells (Figure 15.2B). The DNA vaccine transfer system was first assessed with mouse macrophage cell line, and further evaluation was conducted *in vivo* by infection of

BALB/c mice and cotton rats with the attenuated suicide Δ2 mutant *Listeria* strain carrying GFP-expressing DNA vaccine.[143] Peritoneal macrophage cells isolated from cotton rats infected with the *Listeria*-carrying vaccine exhibited highly efficient expression of GFP, demonstrating that DNA vaccine delivery by the *Listeria* strain is functional *in vivo*. The same system has been also evaluated with primary human DCs and up to 1% of the DCs were shown to express GFP derived from plasmid DNA transferred from the *Listeria* strain.[144,145] Miki and colleagues[72] applied this system for DNA vaccines against *M. tuberculosis*, through construction of the self-destructing attenuated *L. monocytogenes* Δ2 strains carrying eukaryotic expression plasmids for mycobacterial antigen 85 complex molecules (Ag85A and Ag85B) and for the MPB/MPT51 molecule. Immunization of these *Listeria*-carrying DNA vaccines to BALB/c mice intravenously elicited significant protective responses against virulent *M. tuberculosis* challenge.

Although these plasmids were capable of inducing immune responses against *M. tuberculosis in vivo*, they tended to show *in vivo* instability within the carrier *Listeria*. Pilgrim and colleagues[131] modified the *Listeria* system in order to stabilize the plasmid in the *L. monocytogenes* carrier strain. They constructed an *L. monocytogenes* strain that has the chromosomal deletion region encompassing *trpS* gene (encoding tryptophanyl-tRNA synthetase) and also *actA* gene. As *trpS* gene is essential for viability of the bacterium, *trpS*-deleted *Listeria* can maintain only in the presence of a plasmid carrying *trpS* gene. DNA vaccine plasmids were constructed that have *trpS* gene in addition to a listerial autolysis cassette consisting of the *ply118* lysis gene under the control of the *actA* promoter, which is activated in the cytoplasm of infected host cells. No plasmid loss for more than 50 generations of the *Listeria* strain was noted. This new *Listeria*-carrying DNA vaccine permits more efficient cell-to-cell spread than the nonspreading counterparts such as Δ2 mutant *Listeria* strain. However, DNA uptake into the nucleus of host cells is still a major limiting step for optimal gene expression.

Schoen and colleagues[158] described a further improved *Listeria*-carrying vaccine system in which translation-competent mRNA is released directly into the cytoplasm of host cells, leading to immediate translation and production of antigen molecules in host cells. They noticed a much earlier expression of a model protein, enhanced GFP (EGFP), which was detectable within 4 hours of infection, with a much higher number of EGFP-expressing mammalian cells being generated with this novel mRNA delivery system compared to the plasmid DNA delivery system. Loeffler and colleagues[159] also assessed the *in vivo* activation of antigen-specific $CD8^+$ and $CD4^+$ T cells by *L. monocytogenes*-secreted antigen, as well as by *L. monocytogenes* self-destructing, *aroA/B* mutant strain–harboring antigen-encoding plasmid DNA or mRNA. It seems that secretion of a model antigen (OVA) by *L. monocytogenes* yielded the strongest immune responses involving OVA-specific $CD8^+$ and $CD4^+$ T cells. Infection of mice with self-destructing *L. monocytogenes* delivering mRNA for OVA resulted in a significant OVA-specific $CD8^+$ T cell response. However, infection with *L. monocytogenes* delivering OVA-encoding DNA failed to generate specific T cells.

15.4 ANTIVIRAL VACCINES

Recombinant *L. monocytogenes* have been successfully used as biologic vaccine vectors against LCMV, murine influenza virus, and human immunodeficiency virus (HIV) (Tables 15.2 and 15.3). In murine virus infection systems of LCMV and influenza virus, $CD8^+$ T cell responses have been shown to be pivotal for induction of protective immunity through adoptive transfer of virus-specific $CD8^+$ T cells or $CD8^+$ T cell-oriented vaccination studies. In addition to $CD8^+$ T cell responses, *L. monocytogenes* has been shown to induce $CD4^+$ T cell responses. Adjuvanticity of the bacterium directs the $CD4^+$ T cell responses toward Th1-type responses, which are important for protective immunity against viruses.

Murine LCMV infection has been employed as an excellent prototype model of immune responses, especially $CD8^+$ T cell responses, against viruses. Study of murine LCMV infection led to the discovery of MHC restriction.[160] Recombinant *Listeria* vaccines have been developed for

TABLE 15.2
Recombinant *L. monocytogenes* Vaccines for Lymphocytic Choriomeningitis Virus (LCMV) and Influenza Virus

Attenuation	Antigen	Promoter	Immunization Route	Immune Responses	Protection	Ref.
			LCMV			
actA	NP	*hly* (episomal)	i.v.	CD8	+	161
Wild type	NP	*hly* (chromosomal); *actA* (chromosomal)	i.v.	CD8	+	136, 163
Wild type	NP 118–126 (secreted and nonsecreted forms)	*hly* (chromosomal)	i.v.	CD8	ND[a]	127
Wild type	NP	*actA* (chromosomal)	i.v.	CD8	+	162
hly	NP 118–126 (secreted form)	*hly* (chromosomal)	i.v.	CD8	+	58
			Influenza Virus			
hly	LLO-flu NP fusion	*hly* (episomal)	i.v.	CD8 (LLO^+ Listeria); CD4 (LLO^- Listeria)	+	133
hly	LLO-flu NP fusion	*hly* (episomal)	i.v.	CD8	+	164

[a] ND: not determined.

induction of specific $CD8^+$ T cell responses and protective immunity against LCMV. Goossens and colleagues[161] reported an episomal *Listeria* vaccine for LCMV. Immunization of *L. monocytogenes* secreting LLO-NP fusion protein induced LCMV-specific $CD8^+$ T cells and protective immunity in mice against an otherwise lethal intracerebral LCMV challenge. The plasmid was maintained in *L. monocytogenes* for a short time, which appeared adequate for provoking effective antigen-specific $CD8^+$ T cell responses.

Shen and colleagues[136] used a $CD8^+$ T cell epitope in NP of LCMV as a model antigen to examine T cell responses to the secreted or nonsecreted form of the antigen produced by *L. monocytogenes*. Induction of $CD8^+$ T cell responses by both secreted and nonsecreted forms of the antigen was observed. However, only the secreted antigen was capable of being a target of $CD8^+$ T cells. Further, the LCMV-NP-producing recombinant *Listeria* vaccines were protective against subsequent LCMV challenge. Analysis of kinetics of challenged LCMV titer levels in LCMV-NP-producing *Listeria*-immune mice indicates that the LCMV was cleared at 28 days after LCMV challenge, which was later than the virus-cleared timing in LCMV-immunized mice.[162] Duration of LCMV antigen existing *in vivo* was paralleled with the strength of the protective immunity induced by LCMV or by the recombinant *Listeria* vaccine.[163]

Influenza virus NP-expressing *Listeria* vaccine has been also examined for the efficacy to elicit protective immunity against flu virus infection. Ikonomidis and colleagues[133] prepared *L. monocytogenes* secreting a fusion protein consisting of LLO and influenza virus NP, a major protective antigen of influenza virus. Infection of wild-type *L. monocytogenes* strain secreting the LLO-NP fusion protein was capable of processing and presenting the NP antigen to $CD8^+$ T cells through the MHC class I pathway. In contrast, infection of a *hly*-deficient *L. monocytogenes* strain secreting

TABLE 15.3
Recombinant *L. monocytogenes* Vaccines for Human Immunodeficiency Virus (HIV)

Attenuation	Antigen	Promoter	Immunization Route[a]	Immune Responses	Protection	Ref.
Wild type	HIV-1 Gag	*hly* (chromosomal)	i.v., i.p.	CD8	ND[b]	169
hly, actA	HIV-1 gp120	*actA* (episomal)		CD4	ND	173
Wild type	HIV-1 Gag	*hly* (chromosomal)	i.p.	CD4	ND	171
dal, dat	HIV-1 Gag	*hly* (chromosomal)		CD8	ND	174
Wild type	HIV-1 Gag	*hly* (chromosomal)	i.p.	CD8, CD4	+[c]	172
dal, dat	HIV-1 Gag	*hly* (chromosomal)	i.p., i.g.	CD8	+[c]	97
dal, dat	HIV-1 Gag	*hly* (chromosomal)	i.p.	CD8	+[c]	175
dal, dat	HIV-1 Gag	*hly* (chromosomal)	i.p.	CD8	+[c]	176
Wild type	HIV-1 Gag	*hly* (chromosomal)	i.v., i.g., Intrarectal	CD8	ND	178
Wild type	FIV Gag; FIV Env	*hly* (chromosomal) + *CMV-IE* (episomal)	Oral	IgA, IgG	+[d]	179, 180
Wild type	SIV Gag; SIV Env	*hly* (chromosomal)	Oral	CD8	+[e]	100–102[f]
dal, dat (pARS)	HIV-1 Gag	*hly* (chromosomal)	i.v., i.g.	CD8	+[c]	177

[a] i.g., intragastric.
[b] ND: not determined.
[c] Protection against HIV-1 Gag-expressing vaccinia virus in mice.
[d] Protection against FIV in cats.
[e] Protection against SIV in Chinese rhesus macaques.
[f] These three studies used prime-boost strategy (i.e., DNA vaccination followed by *Listeria* vaccination).

the LLO-NP fusion protein was subjected to processing and presenting of the NP antigen to $CD4^+$ T cells, not to $CD8^+$ T cells. As the study involved the episomal *Listeria* vaccine, almost all *Listeria* colonies recovered from organs of mice 48 hours after immunization had lost the plasmid. To avoid the loss of plasmid in recombinant *Listeria*, a *prfA*-deficient *L. monocytogenes* mutant was used as the carrier of a plasmid containing *prfA* and the LLO-NP fusion gene, as mentioned before.[133] Two recombinant *Listeria* vaccines—one strain expressing only an H2-K^d-restricted NP epitope (NP 147-155) and the other strain expressing the full-length NP sequence fused with LLO—helped clear influenza virus infection in mice rapidly, suggesting the feasibility of recombinant *Listeria* vaccines for future influenza virus vaccine.[164]

L. monocytogenes has been well studied as a vaccine platform for HIV.[165,166] A series of studies on *Listeria* vaccines against HIV have used HIV-1 Gag as the target protein. HIV-1 Gag is a viral core protein highly conserved among different virus clades. This protein has been shown to be one of the major CTL antigens in HIV-positive individuals.[167,168] Frankel and colleagues[169] examined the first-generation recombinant *Listeria* carrying HIV-1 Gag or Nef. These genes were inserted into the nonessential *sepA* region of the *Listeria* chromosome, which resulted in three logs of attenuation and induced potent $CD8^+$ T cell responses to the HIV gene products. The HIV-1 Gag-expressing *L. monocytogenes* vaccine was useful for identification of murine CTL epitope of HIV-1 Gag.[170] Following these studies, Mata and Paterson[171] examined $CD4^+$ T cell responses with the same HIV-1 Gag-expressing *L. monocytogenes* immunization. BALB/c and C57BL/6 mice immunized with HIV-1 Gag-expressing *L. monocytogenes* produced $CD4^+$ T cells specific for HIV Gag that secreted high amounts of IFN-γ, indicating Th1 phenotype. Mata and colleagues[172] showed that immunization of HIV-1 Gag-expressing *L. monocytogenes* protected mice against challenge infection by a recombinant vaccinia virus expressing HIV-1 Gag. This protective immunity was mainly attributable to a specific $CD4^+$ T cell subset with a minor contribution of a specific $CD8^+$ T cell subset.