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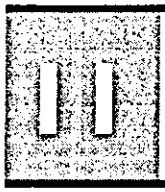

Attenuated bacteria as transfer vehicles of DNA vaccines

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Abstract

DNA vaccines have advantages over other types of vaccines, in relatively easy vaccine design and construction by using recombinant DNA technique, strong induction of cellular immunity, and the relatively low cost. Recently, live attenuated intracellular bacteria have been utilized as the carrier of DNA vaccines in animal models. These bacterial carrier systems have several special features favorable for eliciting effective cellular immunity against pathogens and tumors. The features contain efficient delivery of plasmid DNA to professional antigen-presenting cells, induction of type 1 immune response, and possibility of administration through mucosal routes. Bacteria

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utilized as the vaccine carrier include Salmonella, Shigella, and Listeria. We review here these bacterial DNA vaccine carrier systems reported so far.

Introduction

DNA vaccine offers many advantages over other types of immunizing methods: relatively easy design and construction by using recombinant DNA technique, strong induction of cellular immunity, the chemical stability, the relatively low cost, and so on [reviewed in 1-5].

Major immunization methods for DNA vaccines tried so far include intramuscular injection into the hid leg quadriceps or tibialis anterior and intradermal DNA immunization [6]. In addition, topical application of DNA vaccines [7] has been also reported to be able to show immunization effects. These methods use naked plasmids just resuspended in saline as DNA vaccines. In the intramuscular immunization, primary cells that plasmid DNA is transferred into are considered as myocytes. As the cells are not professional antigen-presenting cells (APCs), the mechanisms of DNA vaccines have been controversial. But, bone marrow-derived APCs have been suggested to be involved in antigen presentation of DNA vaccines [8, 9].

Furthermore, several "carrier"-mediated DNA vaccine administration methods have been reported. They contain liposomes and microparticle encapsulation. These methods are briefly reviewed in [3]. Gene gun injection is the most popular method among the carrier-mediated methods. In this method, plasmid DNA is coated on the small gold particle (the diameter is around 1 μm). DNA binds to gold by electric charge as gold particle is positively charged and plasmid DNA is negatively charged. The gold particle was then coated inside of plastic tube. After the tube is cut to small pieces and applied to "gene gun" apparatus. Finally, gold particle coated with plasmid DNA is injected against skin of animals by the pressure of helium gas and goes into host cells directly. It is of interest that gene gun DNA immunization requires 100- to 1,000-fold less DNA than muscle DNA inoculation to generate equivalent antibody responses (several μg per shot in the case of gene gun method) [10, 11]. In addition, gene gun DNA immunization appeared to bring about highly reproducible and reliable results in antibody production, induction of specific cytotoxic T-lymphocytes (CTL) and interferon (IFN)-γ production from immune splenocytes [12]. It has been suggested that muscle DNA immunization tends to raise predominant type 1 helper T (Th1) responses, while gene gun DNA immunization produces type 2 helper T (Th2) responses [13]. These differences are considered to be mainly due to the differences of plasmid amounts used for vaccination, which may affect amounts of antigen produced from the plasmids and amounts of CpG motifs present in plasmid DNA vaccines. The attenuated bacteria-carrier method is one of these "carrier"-mediated DNA vaccine systems [14].

Structure of live bacteria-carrying DNA vaccines

Fig. 1 illustrates a typical plasmid utilized for live bacteria-carrying DNA vaccines. The structure of plasmid DNA for live bacteria-carrying DNA vaccine system is basically same with that of plasmid DNA for naked DNA vaccination. Namely, DNA vaccines are composed of (1) an antigen-encoding gene whose expression is driven by (2) a strong eukaryotic promoter such as cytomegalovirus immediate-early promoter/enhancer (CMV IE enhancer/ promoter). And the plasmid possesses (3) a polyadenylation termination sequences such as the sequence derived from simian virus 40 (SV40) or bovine growth hormone (BGH) gene and (4) a selective marker such as ampicillin resistance gene to facilitate selection of *Escherichia coli* having the plasmid. In addition, plasmids for DNA vaccines should contain special nucleotide sequences for enhancing the immunogenicity; an unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide with appropriate flanking regions. In mice, the optimal flanking region is composed of two 5' purines and two 3' pyrimidines [15]. Ampicillin resistance gene contains the CpG, but kanamycin resistance gene does not have it [16]. The CpG motif stimulates the innate immune system through Toll-like receptor (TLR) 9 to produce a series of

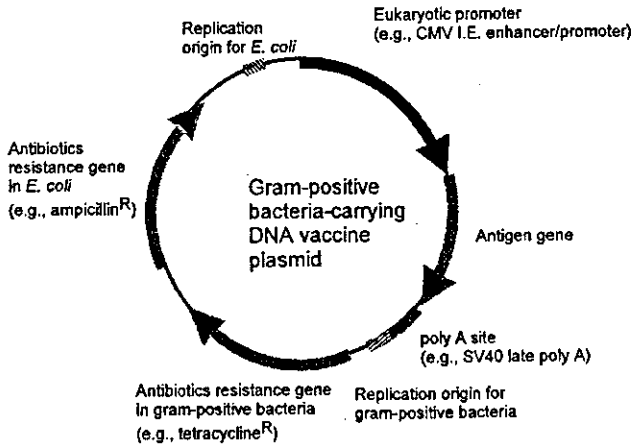

Figure 1. Schematic drawing of a typical plasmid for DNA vaccine using gram-positive bacteria as the carrier. The plasmid should contain a replication origin for *E.coli* and also a replication origin for the carrier gram-positive bacterium, antibiotic resistance genes for *E.coli* and the carrier gram-positive bacterium, and a sequence that encodes the antigen of interest, which is driven by a strong eukaryotic promoter and is followed by termination and polyadenylation signal sequences.

immunomodulatory cytokines such as interleukin-12 and interferon (IFN)-γ, which promote the development of Th1 cells [17-19].

In addition of these features, plasmid DNA for gram-positive bacteria-carrying DNA vaccines should contain an origin of replication for the carrier gram-positive bacteria as an origin of replication for *E. coli* in plasmids used routinely in laboratories does not work in gram-positive bacteria. In the case of *Salmonella* and *Shigella* carriers, an origin of replication for *E. coli* may work as they belong to the same family *Enterobacteriaceae*. All the replication origins in gram-positive bacteria do not necessarily work in all gram-positive bacteria. For example, a replication origin of pAMβ1 derived from *Enterococcus faecalis* [20] was reported to be the most adequate for stable replication in *L. monocytogenes* [21].

Intracellular bacteria used for DNA vaccine carrier

Bacteria used for DNA vaccine carrier have to be weak in terms of pathogenicity (attenuation), but should keep immunogenicity. The attenuated mutation of pathogenic bacteria is usually made by metabolic attenuation (auxotrophy). In addition, further attenuation of bacteria in vivo will be achieved by an inducible autolytic mechanism or simply from treatment with antibiotics. Plasmids maintained in attenuated bacteria are then liberated and transfer into the nucleus of the host cells, leading to expression of the encoded protein. Usually, immunogenicity of vaccines is related to the "invasiveness" of the bacterial carrier. In general, increasing the invasiveness improves the immunogenicity.

Bacteria utilized as this type of the vaccine carrier contain *Salmonella* and *Shigella*, as well as *Listeria*. They are categorized as intracellular bacteria based on their localization in the host. These bacteria are "facultative" intracellular bacteria as they are also able to survive outside of host cell. In contrast, *Chlamydia* and *Rickettsia* cannot survive outside of host cells like viruses. They are therefore called "obligate" intracellular bacteria. Intracellular bacteria are divided to three different groups depending on its intracellular niche [22, 23]. They are, (1) cytoplasmic bacteria, which exit the phagosome and reside in the host cell cytosol; (2) intralysosomal bacteria, which persist in acidic, hydrolytic compartments that interact with the endosomal network of the host; (3) Intravacuolar bacteria, which persist in nonacidic vacuoles that exhibit modified or little interaction with the endosomal system of the host (Table 1).

(1) Intracytosolic bacteria

A few bacteria have evolved mechanisms that allow them to avoid the potentially hostile environment of the endosomal and lysosomal network of the host cells by escaping into the host cell cytosol. This is the most useful feature

Table 1. Intracellular bacteria

Intracytosolic bacteria
Listeria monocytogenes, Shigella flexneri**
Rickettsia prowazeki
Intralysosomal bacteria
Salmonella typhimurium, Salmonella typhi**
Coxiella burneti
Intravacuolar bacteria
(inside arrested phagosome)
Mycobacterium spp. (*M. tuberculosis, M. bovis, M. avium*)
Nocardia asteroides
(inside sequestered phagosome)
Legionella pneumophila, Chlamydia trachomatis

Asterisks indicate bacteria utilized as carrier for DNA vaccines.

as DNA vaccine carrier. DNA vaccine-carriers which belong to this group of bacteria release DNA vaccine plasmids after the lysis in the host cell cytoplasm. They have developed strategies to escape into the host cell cytosol, replicate in the cytosol, and spread from one infected cell to an adjacent one. *Shigella* and *Listeria* have been used as carrier for DNA vaccines. *Rickettsia* is a genus for very small obligate intracellular bacteria and belongs to this group, but has not been used for this purpose.

i) *Shigella*: Four species consisting of more than 45 O antigen-based serotypes have been described. They are *Shigella dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. *S. sonnei* is the most common cause of shigellosis in the industrial world, and *S. flexneri* is the most common in undeveloped countries. *Shigella* appears unable to attach to differentiated mucosal cells, rather, they first attach to and invade M (microfold) cells located in Peyer's patches. Then, the bacteria go into underlying macrophages. Although *Shigella* is often categorized as intracellular bacteria, *Shigella* intrudes host cells transiently [24]. It was observed that the ability of these bacilli to replicate inside cells in culture correlated not with the production of Shiga toxin but with the presence of a 140-MDa plasmid, pWR100 [25].
ii) *Listeria*: *Listeria* is the causative agent of listeriosis and has emerged recently as the model bacterium for analyzing the mechanisms of survival of intracytosolic bacteria [26-28]. Like *Shigella*, *Listeria monocytogenes* escapes from the vacuole into the cytoplasm shortly after entry into its host cell. *L. monocytogenes* possesses a hemolysin named listeriolysin O (LLO) which is a well-characterized molecule with membrane-disrupting capability. LLO is a thiol-activated cytolysin that belongs to a family of homologous proteins present

in several pathogenic bacilli, such as perfingolysin (PFO) derived from *Clostridium perfringens* and streptolysin O (SLO) from *Streptococcus pyogenes*.

(2) Intralysosomal bacteria

Salmonella belongs to this group. More than 2,000 unique serotypes of *Salmonella* have been described. Analysis of DNA homology revealed that the genus consists of a single species *(S. enterica)* subdivided into seven subgroups. *Salmonella* penetrates through M cells into the Peyer's patches, in which they can be phagocytosed by the underlying macrophages [29]. The fine nature of *Salmonella*-containing vacuoles has been controversial depending on the cells *Salmonella* infected and investigators, but the vacuole has the characteristics consistent with the vacuoles being late endosomal or lysosomal in character. Although the acidification of the vacuoles was partially reduced in the vacuoles containing live *Salmonella*, but the pH of the vacuoles containing live bacteria was still relatively acidic. These vacuoles showed unrestricted fusion with lysosomes, as demonstrated by the acquisition of lysosomal contents. *Salmonella* vacuoles in macrophages are subjected to be acidified by the fusion with lysosomes. Interestingly, it is reported that blocking the acidification of phagosomes with bafilomycin A, a vacuolar ATPase inhibitor, reduced the viability of the bacilli. This suggests that a drop in pH is required for full induction of intracellular survival strategies in *Salmonella*.

Yersinia has been also used as the carrier of DNA vaccines. The genus *Yersinia* consists of 11 species. Among them, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are the best known human pathogens. Attenuated *Y. enterocolitica* mutant strains have been shown to deliver DNA in vitro and in vivo. In contrast to *Shigella* infections, *Yersinia* does not replicate in the M cells in Peyer's patches, but rather pass through to the underlying tissue. The bacillus is then engulfed by macrophages, carried to the mesenteric lymph nodes, and replicate. *Yersinia* has been considered to be among facultative intracellular bacteria as *Yersinia* intrudes host cells transiently like *Shigella* [24]. However, they have been reported to replicate predominantly, if not exclusively, in extracellular sites in vivo [30].

(3) Intravacuolar bacteria

Bacteria like *Mycobacterium* and *Nocardia* block the normal maturation procedure of phagosomes and fail to fuse with lysosomes. Vacuoles containing *Mycobacterium* did not acidify below pH6.2 to 6.5 and showed a paucity of the vacuolar proton-ATPase responsible for acidification of endosomal and lysosomal compartments. However, the vacuoles have been reported to possess lysosome-associated membrane protein-1 (LAMP-1), class I and II MHC molecules, cathepsin D, and transferrin receptor. This group of bacteria has not been used as carrier for DNA vaccines.

Advantages and disadvantages of attenuated live bacterial vaccines

Among possible vaccine systems, live attenuated bacteria are promising vaccine candidates for induction of protective immunity against infectious diseases and tumors. Advantages of the attenuated live bacteria DNA vaccine systems are as follows.

(1) Possible mucosal route of immunization

Many human infectious diseases are initiated at mucosal surfaces. Live attenuated bacteria-carrying DNA vaccine can be administered by a mucosal route. The routes contain oral, intranasal, intragastric, intravaginal, and rectal routes. Administration of the vaccine through these routes mimics the immune response elicited by natural infection and can lead to long-lasting protective mucosal and systemic responses. Moreover, vaccination via a mucosal route is associated with lower rates of side effects and in many cases lower delivery costs. Two oral live bacterial strains are licensed for human use at present. These are *Salmonella enterica* serovar Typhi (*S. typhi*) Ty21a [31] and *Vibrio cholerae* CVD103-HgR [32]. They have been used against typhoid fever and cholera, respectively. *S. typhi* Ty21a has been used as a carrier for DNA vaccines [33]. However, murine model of oral *Listeria* DNA vaccine carrier system may have some problem. For *L. monocytogenes*, two surface proteins, the internalins A and B, are responsible for invasion of non-phagocytic cells and seem to determine the specificity of the cell type infected. Mouse E-cadherin, which serves as a receptor for internalin A, is reported to have a glutamine to proline substitution [34, 35].

(2) Propensity to infect APCs

Usually, pathogens invading into host are phagocytosed and digested in phagocytic cells such as macrophages. In contrast, intracellular bacteria misuse them as a habitat. *Salmonella* and *Listeria* show a strong preference for macrophages [23]. Furthermore, these bacteria have been also shown to infect dendritic cells, the most powerful APCs [36, 37]. Therefore, facultative intracellular bacteria should be ideal carriers for heterologous antigens and the elicitation of cellular immune responses. Especially, the mucosal route of immunization of attenuated bacteria-carrier DNA vaccines may lead to efficient antigen presentation in the mucosal site, a major entry site of pathogens.

(3) Ease of genetic manipulation

Salmonella, *Shigella*, and *Listeria* are able to be relatively easily transformed with conventional DNA vaccine vectors, although they are more difficult than *E. coli*.

Methods for growing and processing these bacteria have been established. In addition, the amount of additional DNA that can be cloned into bacterial plasmids is several orders of magnitude larger than the current viral vectors can accommodate.

(4) Adjuvanticity of carrier bacteria

Cell-surface moieties of bacteria such as lipopolysaccharide (LPS) in gram-negative bacteria and lipoteichoic acid in gram-positive bacteria are recognized by host cells as "danger" signal and stimulate innate immune responses via the TLRs, pattern recognition receptors, on host cells. In addition, unmethylated CpG sequence abundant in bacterial genomic DNA may stimulate innate immunity through TLR9-mediated signal. Live attenuated bacteria act as "natural adjuvants", thereby attracting cells involved in innate immunity and promoting APC maturation. Thus, APCs can be activated to express the necessary co-stimulatory molecules by these stimuli. In addition, the Th1-like pattern of cytokines is induced upon infection of macrophages with these bacteria, including interleukin (IL)-12, IFN-γ, and tumor necrosis factor (TNF)-α, which may subsequently enhance antigen presentation by these infected cells, and also bias the ensuing immune response in the direction of cell-mediated immunity.

(5) Possible amplification of DNA vaccine plasmids in vivo

The low efficiency of traditional naked DNA vaccination can be due to the fact that limited amounts of DNA vaccine plasmids in vivo. Live attenuated bacteria harboring DNA vaccine plasmid allow the plasmid replicates inside.

(6) Simplicity of handling and stock

Bacteria-carrier DNA vaccine system does not require DNA purification which is an indispensable step for naked DNA vaccination. And the bacteria can be stocked by lyophilization as currently used *M. bovis* BCG vaccine. In addition, bacteria are easily controlled by commonly used antibiotics, a situation that is usually not available with viral vectors.

On the other hand, live bacteria-carrier DNA vaccine system has some possible issues. The major biosafety issues related to naked DNA vaccination are also the issues of bacteria-carrying DNA vaccines. They include, (1) potential risk of chromosomal integration, (2) the spread to, and long-term persistence of the plasmid in multiple tissues; (3) the induction of tolerance to the immunization antigen; (4) the risk of autoimmune disease by elicitation of anti-DNA antibodies. In addition, attenuated bacterial carrier system has (5) possibility of reversion to toxic phenotype. For DNA vaccine carrier, the appearance of wild-type bacteria is major issues especially when immunized

into immunocompromized hosts. In order to prevent this situation, a vaccine strain should contain appropriate attenuation in genes that are essential for survival in the host, and more than two genetically unlinked attenuating mutations. But, too much attenuation may reduce immunogenicity of the vaccine. Therefore, the balance between attenuation (safety) and keeping immunogenicity of vaccines is one of important issues for clinical adaptation of vaccine candidates. Furthermore, (6) bacterial restriction and modification system may hamper the maintenance of plasmids introduced into the bacteria. A bacterium contains several systems that protect its host DNA from exogenous DNA. 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 ubiquitously used in laboratories have mutations in these systems by genetic manipulation. However, *Salmonella*, *Shigella*, and *Listeria* strains used as DNA vaccine carrier 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. (7) Intrinsic immunogenicity of viable bacteria itself is another issue. Live attenuated bacteria composed of a bunch of proteins and lipid, sugar, and so on, which are themselves immunogenic in host cells. So, repeated immunization of live bacterial vaccine may cause rapid elimination of live bacterial vaccine [38, 39]. Similar situation have been discussed especially with adenovirus-based vaccines. One of strategies for this possibility is that combination of different vaccination methods. For example, first immunization is by naked DNA vaccination, then boosting with live bacterial vaccine. Alternatively, different carrier bacteria are able to use for each immunization step. However, several reports indicate that prior immunologic experience to *Salmonella* enhance subsequent immune responses by boost immunization [40-42]. The exact reason of these conflicting results has not been clear.

Shigella delivery of plasmid DNA

First reported DNA vaccine-carrying bacterium belongs to *Shigella*. Sizemore et al. [43, 44] showed that *S. flexneri* 2a 15D harboring a plasmid expressing a *lacZ* reporter gene controlled by CMV IE enhancer/promoter

elicited modest antibody and cellular immune responses against the reporter protein. *Shigella* strain 15D (15D is a derivative of wild-type *S. flexneri* 2a strain 2457T) harbors a deletion mutation in the *asd* gene encoding aspartate β-semialdehyde dehydrogenase, an essential enzyme that is required to synthesize the bacterial cell wall component diaminopimelic acid (DAP). The DAP auxotrophs retain invasiveness for mammalian cells, yet lyse rapidly in the absence of DAP supplementation in vivo [45]. The use of an invasive yet non-replicating attenuated vector such as *S. flexneri* 15D may be suitable for delivering plasmid DNA vaccines to mucosal lymphoid tissues. These results are supported by experiments in mice intranasally immunized with strain 15D expressing measles virus proteins by Fennely et al. [33]. They showed that mice vaccinated with strain 15D harboring plasmid vectors encoding different measles virus antigens (envelope protein and nucleoprotein) induced a vigorous measles virus antigen-specific response. They observed the production of measles virus protein-specific $CD8^+$ T-lymphocyte and IFN-γ responses, as well as the modest production of specific serum antibodies.

Vecino et al. [46] reported that in a murine intranasal immunization model, *S. flexneri* strain 15D harboring a eukaryotic expression plasmid for human immunodeficiency virus (HIV) gp120 induced HIV-specific IFN-γ-producing $CD8^+$ T cells among splenocytes more efficiently than either Δ*aroA S. typhimurium* strain SL7207 or *S. typhi* Ty21a harboring the same DNA vaccine.

Listeria delivery of plasmid DNA

The ability of *L. monocytogenes* to enter the host cytosol after phagocytosis and deliver plasmid DNA directly to the cytoplasm makes it an attractive DNA delivery candidate to induce cellular immune responses [26-28].

Hense et al. [47] evaluated *Listeria* as vehicles for gene transfer using a variety of cell lines. They observed gene transfer to host cells after treating host cells infected with plasmid-carrying *Listeria* with tetracycline, an antibiotic that is only bacteriostatic. They speculate that the metabolic block by tetracycline treatment makes these bacteria susceptible to cellular defense and degradation mechanisms that otherwise would be harmless to the bacteria. They reported that bacterial properties required for delivery of the eukaryotic expression plasmids were strictly dependent on the ability of the bacteria to both invade eukaryotic cells and egress from the vacuole into the cytosol of the infected host cells. In addition, they showed that macrophage-like cells or primary, peritoneal macrophages were almost refractory to *Listeria*-mediated gene transfer.

Dietrich et al. [48] reported a DNA vaccination system of an attenuated self-destructing *L. monocytogenes* strain. They demonstrated the feasibility of the system in cell culture system. They used a deletion mutant of *L. monocytogenes* Δ2 that lacks the entire lecithinase operon including the virulence-associated genes

actA, *mpl*, and *plcB* [49]. This strain can infect macrophages and replicate in the cytoplasm, but cannot spread to adjacent cells. This attenuated mutant was introduced with a plasmid containing the gene for lysis protein PLY118 of the listerial bacteriophage A118. The *ply118* gene expression was controlled by *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. 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 (Fig. 2). Recently, we applied this system for DNA vaccines against *M. tuberculosis* [50]. We constructed self-destructing attenuated *L. monocytogenes* Δ2 strains carrying eukaryotic expression plasmids for mycobacterial antigen 85 complex (Ag85A and Ag85B) and MPB51 molecules. Intravenous immunization of these *Listeria*-carrying DNA vaccines to BALB/c mice elicited significant protective responses against virulent *M. tuberculosis*.

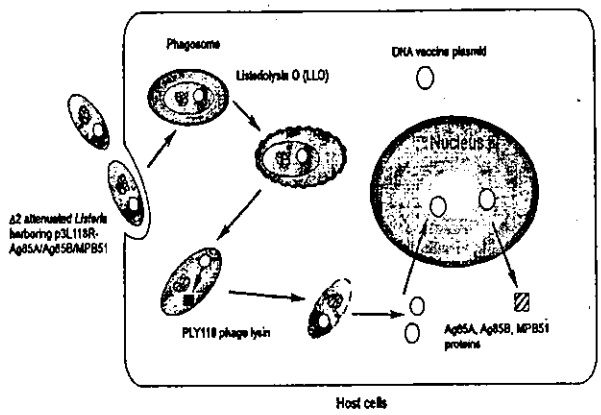

Figure 2. Schema of Δ2 *L. monocytogenes* Ag85A, Ag85B, MPB51 DNA vaccine carrier system. *Δ2 L. monocytogenes* strain can infect macrophages and replicate in the cytoplasm, but cannot spread to adjacent cells. P3L118R-Ag85A, -Ag85B, or MPB51 plasmids contain the gene for lysis protein PLY118 (*ply118*) derived from listerial bacteriophage A118. *ply118* expression was controlled by *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. Autolysis of the *L. monocytogenes* mutants apparently release the plasmid DNA into the host cell cytoplasm, allowing expression of *Ag85A*, *Ag85B*, or *MPB51* gene in the host cells.

Although we were able to induce immune responses against *M. tuberculosis* in vivo, these plasmids tend to be lost from carrier *Listeria* in vivo [51]. Pilgrim et al. [21] modified the *Listeria* system in order to stabilize the plasmid in the *L. monocytogenes* carrier strain. They constructed an *L. monocytogenes* strain which has the chromosomal deletion region compassing *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 plasmid carrying *trpS* gene.

They constructed DNA vaccine plasmids having *trpS* gene in addition to listerial autolysis cassette consisting of the lysis gene of phage A118 (*ply118*) under the control of the *actA* promoter which is activated in the cytosol of infected mammalian host cells as mentioned before. They reported that no plasmid loss for more than 50 generations of the *Listeria*. This new *Listeria*-carrying DNA vaccine allow cell-to-cell spread, which was found to be much more efficient in DNA delivery than the nonspreading counterparts like Δ2 listerial strain [21].

Salmonella delivery of plasmid DNA

Attenuated mutants of *Salmonella enterica* serovar Typhi (*S. typhi*) and *S. enterica* serovar Typhimurium (*S. typhimurium*) have been extensively studied as vaccine carriers. Most knowledge on bacteria-mediated gene transfer has been acquired using attenuated *S. typhimurium* as carrier. The attenuated *S. typhimurium* strain that have been most often used is *S. typhimurium aroA* mutant [52], which interferes with the biosynthesis of aromatic amino acids. Furthermore, investigators reported a variety of new mutants whose attenuation levels are comparable to, but more immunogenic than *aroA* mutant [53, 54]. DNA vaccine-carrying *Salmonella* will die in the host cell vacuoles due to metabolic attenuation and release DNA vaccine plasmids. These plasmids cross the vesicular membrane and reach the cell nucleus of the host cells where they are expressed.

Darji et al. [55] reported an orally administered attenuated *S. typhimurium aroA* mutant carrying plasmids containing the coding sequences of β-galactosidase gene of *E. coli*, or truncated forms of ActA or listeriolysin of *L. monocytogenes* driven by eukaryotic promoters, induce efficient humoral and cellular immune responses. Immunization of *Salmonella* carrying a listeriolysin-encoding expression plasmid elicited a protective immunity against a lethal dose of *L. monocytogenes* challenge. The observation that *Salmonella* acts as a delivery system for DNA vaccines was unexpected, since the bacterium does not normally access the host cytosol as *Listeria* and *Shigella* do. However, a pilot study by Aggarwal et al. [56] reported recombinant *Salmonella* vaccine vectors are capable of inducing $CD8^+$ CTL responses against antigens although their

system was not a DNA vaccine carrier system. Recently, Perrin et al. [57] reported that a small population of *Salmonella* escape *Salmonella*-containing vacuole and are released into the cytosol. Alternatively, as *Salmonella* is reported to induce apoptosis when it enters macrophages [58], bystander dendritic cells may capture DNA vaccine plasmid through phagocytosis of *Salmonella*-infected apoptotic cells [59].

In addition, Brunham and Zhang [60] reported that oral administration of *S. typhimurium* 22-11 carrying *Chlamydia trachomatis* major outer membrane protein-expression plasmid elicited a protective response against *C. trachomatis* infection. Many reports have also accumulated on oral *Salmonella* DNA vaccines against tumors. All studies so far were performed in mice and exclusively with *S. typhimurium* Δ*aroA* (SL7207) as a carrier. Paglia et al. [36] reported that oral administration of *S. typhimurium aroA* mutant (SL7207) carrying *lacZ*-expressing plasmid partially protected against an aggressive fibrosarocoma expressing β-galactosidase protein. They reported also that oral administration of *S. typhimurium* harboring plasmid DNA encoding green fluorescent protein gave rise to fluorescent dendritic cells with extremely high efficiency (as many as 50% of the $CD11c^+$ splenic dendritic cells), indicating that plasmid DNA was expressed in most potent APCs, dendritic cells. In addition, *S. typhimurium* Δ*aroA* (SL7207) carrying expression plasmids for cytokines have been reported. Plasmids encoding IL-4, IL-18, IL-12, and human granulocyte-macrophage colony-stimulating factor (hGM-CSF) were delivered with attenuated *S. typhimurium* [61, 62].

Other attenuated *Salmonella* includes *S. typhi*. *S. typhi* Ty21a, a *galE* mutant strain, is the strain approved for humans and has been used for decades [31]. The mutation interferes with the production of Vi polysaccharide, which leads to lysis of the bacterial cells and may enhance plasmid delivery in vivo. Fennelly et al. [33] showed that intraperitoneal administration of *S. typhi* Ty21a carrying measles nucleoprotein expression plasmid is capable of eliciting antigen-specific CTL response. Another example is the delivery of DNA vaccines with *S. typhi* Δ*guaBA*. Pasetti et al. [63] reported that *S. typhi* Δ*guaBA* strain CVD915 carrying eukaryotic or prokaryotic expression plasmid for fragment C of tetanus toxin is superior to intramuscular naked DNA vaccine administration in antigen-specific antibody and T-cell responses.

In addition to oral *Salmonella* DNA vaccine administration, the nasal route of administration has been also examined. Darji et al. [64] compared oral and nasal administration of *Salmonella* harboring a eukaryotic expression plasmid encoding β-galactosidase. They showed both routes could induce systemic T cell responses but nasal administration was clearly inferior to oral administration possibly due to the lower number of bacteria that could be applied nasally. Interestingly, oral administration induced antibodies in the gut and not in the

lung and nasal administration induced antibodies in the lung but not in the gut. Shata et al. [65] reported that an oral *Salmonella*-carrying DNA vaccine expressing the HIV-1 gp120 envelope protein induced the production of $CD8^+$ T-cells in both mucosal and systemic lymphoid tissues.

Several investigators have improved *Salmonella* carrier by introducing genes conferring invasiveness or autolysis functions. Jain and Mekalanos [66] reported *Salmonella* strain allowing programmed lysis, taking advantages of expression plasmid for λ phage *S* and *R* genes under the control of an arabinose-inducible promoter. In addition, the LLO gene of *L. monocytogenes* into an *S. typhimurium* Δ*aroA* strain [67, 68] resulted in enhanced plasmid delivery.

Delivery of plasmid DNA by other bacteria

Al-Mariri et al. [69] reported an attenuated *Y. enterocolitica* strain harboring DNA vaccines encoding the Brucella *abortus* T-cell antigens, bacterioferritin or p39. The oral immunization resulted in Th1-type responses against both antigens. Optimal protection against *Brucella* challenge was achieved by delivering the DNA vaccines with a *Yersinia* strain expressing an O-polysaccharide that is cross-reactive with *Brucella* lipopolysaccharide.

Bacteria which are not categorized as intracellular bacteria, *E. coli* and *Vibrio cholerae*, have also examined as DNA vaccine carrier. Courvalin et al. [70] examined the laboratory strain of *E. coli* K12 for their ability to transfer plasmid DNA to eukaryotic cells. As wild-type *E. coli* is not invasive, it was transformed with the virulence plasmid of *S. flexneri*. Such *E. coli* was capable of transferring DNA to the eukaryotic cells. Subsequently, same authors and another group made attenuated *E. coli* K12 mutants harboring the invasion gene from *Y. pseudotuberculosis* [71] or listeriolysin gene from *L. monocytogenes* [72] and showed that *E. coli* K12 is able to transfer genetic materials into nonprofessional phagocytic cells and macrophages if the ability to invade the host cells is obtained. Jain and Mekalanos [66] reported *V. cholerae* strain is able to deliver plasmid DNA if the bacteria carry self-lysis apparatus using phage λ phage lysis system similar to the aforementioned *Salmonella* system. *V. cholerae* has a tendency to produce much DNase than either *E. coli* or *S. typhimurium*. So, the authors recommended DNase-defective strain to stable delivery of plasmid DNA into eukaryotic cells.

In addition, *Agrobacterium tumefaciens* was reported to be able to transfer expression cassettes even without invading the target cells. This bacterium is basically a soil phytopathogen that elicits neoplastic growths on the host plant species. Kunik et al. [73] reported that the bacterium attaches to and genetically transforms several types of human cells including HeLa cells, human embryonic kidney (HEK) 293 cells, and pheochromocytoma PC12 neuronal cells by introducing and integrating the bacterial tumor-inducing (Ti) plasmid.

BCG vaccine (*Mycobacterium bovis* Bacille Calmette-Guerin) has been used for live vaccine against *M. tuberculosis* for a long time since 1920s [74]. Although there is controversy upon the vaccine efficacy especially for adult tuberculosis [75], BCG is associated with extremely low frequencies of serious complications. And BCG has been reported to exhibit adjuvant activity mainly due to the lipid and DNA moiety. Although a variety of recombinant BCG which express heterologous antigens have been reported, the report in which BCG is utilized as DNA vaccine carrier has not been reported.

Strategies to improve immune responses

A variety of studies has been performed to enhance immune responses by naked DNA vaccination [reviewed in 5]. These strategies are also applicable to bacteria-carrying DNA vaccines. Such studies using bacteria-carrying DNA vaccines have been reported.

(1) Prime-boost immunization methods

As examined with combination of vaccination methods of naked DNA vaccination and viral vector [76, 77], combination of different vaccination methods has been examined. Shata and Hone [78] reported *S. flexneri* 2a Δ*aroA* Δ*iscA* delivery of the gp120 DNA vaccine, to develop a prime-boost strategy aimed at the induction of HIV-1 neutralizing antibodies and specific $CD8^+$ T cells. They used a strategy based on priming by the *Shigella*-based DNA vaccine construct, followed by boosting with a vaccinia expressing HIV envelope protein (*env*), which induced more intense $CD8^+$ T-cell responses than either vaccine given alone. DeVico et al. [79] reported a prime-boost regimen consisted of a *Shigella*/DNA vaccine prime, followed by a boost with *S. typhimurium* Δ*aroA* mutant expressing gp120, which yielded strong gp120-specific $CD8^+$ T-cell responses. Xu et al. [80] reported another regimen, a naked HIV-1-*gag* DNA vaccine prime, followed by boosting with *S. flexneri* Δ*rfbF* carrying the same vector. Zoller and Christ [81] showed that oral vaccination with *S. typhimurium* carrying β-galactosidase-expression plasmid followed by intravenous transfer of β-galactosidase protein-loaded dendritic-cell immunization provided the optimal schedule for rejecting renal cell carcinoma line RENCA transfected with *lacZ* gene.

(2) Addition of adjuvants

Live bacteria are also used for the delivery of expression plasmids encoding immunomodulatory cytokines in addition to genes for the target antigen molecules [61, 62]. Delivery of two different plasmids encoding an antigenic molecule and a cytokine can be achieved by two different origins of replication,

allowing for co-delivery by a single carrier strain, or delivery using two separate live bacteria strains.

(3) Modification of DNA vaccine plasmids

Several reports have been published to improve the immunogenicity of attenuated bacteria-carrier DNA vaccines. These strategies have been reviewed nicely by Dietrich et al. [82]. They include (1) addition of post-transcriptional regulatory acting RNA element derived from Woodchuck hepatitis B virus to improve gene expression by modification of polyadenylation, RNA export, or translation [83]; (2) addition of ubiquitin to antigen for efficient antigen processing [84, 85]; (3) construction of gene for antigen molecule (gp100) fused with invariant chain molecule, which has endosome/lysosome targeting signal, to facilitate MHC class II-mediated antigen presentation [86].

Summary

DNA vaccines utilizing attenuated bacteria carrier have been examined. So far, *Shigella flexneri*, *Listeria monocytogenes*, and *Salmonella typhimurium and S. typhi* have been mainly utilized for carrier of DNA vaccines. *Shigella* and *Listeria* belong to intracytosolic bacteria and *Salmonella* belongs to intralysosomal bacteria. They have been reported to be able to transfer plasmid DNA to eukaryotic cells. Advantages of bacteria-carrying DNA vaccines contain (1) possible mucosal route of immunization, (2) propensity to infect professional APCs, (3) ease of genetic manipulation, (4) adjuvanticity of carrier bacteria, (5) possible amplification of DNA vaccine plasmids in vivo, and (6) simplicity of handling and stock of the vaccine. A variety of strategies have been examined to enhance immune responses by bacteria-carrier DNA vaccines such as (1) prime-boost vaccine protocol, (2) addition of adjuvants, (3) modification of DNA vaccine plasmids.

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