Mucosal Vaccination and Therapy with Genetically Modified Lactic Acid Bacteria

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Abstract

Lactic acid bacteria (LAB) have proved to be effective mucosal delivery vehicles that overcome the problem of delivering functional proteins to the mucosal tissues. By the intranasal route, both live and killed LAB vaccine strains have been shown to elicit mucosal and systemic immune responses that afford protection against infectious challenges. To be effective via oral administration, frequent dosing over several weeks is required but new targeting and adjuvant strategies have clearly demonstrated the potential to increase the immunogenicity and protective immunity of LAB vaccines. Oral administration of *Lactococcus lactis* has been shown to induce antigen-specific oral tolerance (OT) to secreted recombinant antigens. LAB delivery is more efficient at inducing OT than the purified antigen, thus avoiding the need for purification of large quantities of antigen. This approach holds promise for new therapeutic interventions in allergies and antigen-induced autoimmune diseases. Several clinical and research reports demonstrate considerable progress in the application of genetically modified *L. lactis* for the treatment of inflammatory bowel disease (IBD). New medical targets are on the horizon, and the approval by several health authorities and biosafety committees of a containment system for a genetically modified *L. lactis* that secretes Il-10 should pave the way for new LAB delivery applications in the future.

INTRODUCTION

Several species of lactic acid bacteria (LAB) have been explored as mucosal delivery vehicles for vaccines and therapeutic molecules. This includes *Streptococcus gordonii* and members of the dietary group of LAB, including *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Lactococcus lactis*, which have a generally regarded as safe (GRAS) status owing to their longstanding use in human food fermentations and products. The LAB have limited biosynthetic abilities and require preformed amino acids, B vitamins, purines, pyrimidines, and (usually) a sugar as a carbon and energy source, which is fermented to produce lactic acid as a common end product. These nutritional requirements restrict their habitats to those in which the required compounds are abundant. Nevertheless, LAB occupy a variety of niches, including milk, plant surfaces, the oral cavity, the gastrointestinal (GI) tract, and the vagina of vertebrates. In the human ileum and jejunum, lactobacilli and streptococci are highly represented (10^3–10^5 organisms per gram of luminal contents). The more complex colonic microbiota comprises around 10^{11} bacteria per gram, with streptococci and lactobacilli present at relatively moderate densities (10^6–10^8 per gram) (Hayashi et al. 2005, Vaughan et al. 2002).

The mucosal delivery of vaccines for large-scale immunization programs is an explicit goal of the World Health Organization (WHO) for economical, logistical, and safety reasons. Additionally, oral (mucosal) vaccines have the potential to elicit antigen-specific secretory immunoglobulin A (sIgA) responses at mucosal surfaces, which can neutralize viruses or toxins and inhibit colonization by enteric microbes. It is now recognized that many of the existing vaccines could be improved by use of a mucosal delivery system that can elicit both antigen-specific sIgA and effective systemic immune responses (Lavelle & O'Hagan 2006, Mannam et al. 2004, Neutra & Kozlowski 2006). In reality, this objective is not easy to achieve via a single oral administration of a vaccine except in the case of an attenuated pathogen, thus the development of effective mucosal delivery systems remains an active area of research. The mucosal delivery of therapeutic molecules using LAB may also be a cost-effective approach for the treatment of mucosal-associated diseases, with the potential to lower the therapeutic dose and reduce or avoid possible side effects due to systemic administration.

To evoke mucosal responses, a delivery system is needed to avoid degradation of the antigen, promote uptake of the antigen in the GI tract, and stimulate adaptive rather than the tolerogenic immune responses seen in feeding studies with soluble antigens (Lavelle & O'Hagan 2006, Neutra & Kozlowski 2006). Another reason for using the dietary group of LAB as mucosal delivery vehicles stems from their long and safe association with humans and their food. This obviates the need to attenuate the bacterial vehicle to avoid reactogenicity (Tacket & Levine 2007) but nevertheless provides a vehicle that can potentially survive transit through the intestinal tract and produce recombinant vaccines or therapeutic molecules in situ. Another advantage of using LAB as mucosal delivery vehicles is that they can be engineered to express multiple proteins and other molecules, e.g., the expression of the type 3 capsule biosynthesis genes of *Streptococcus pneumoniae* in *L. lactis* produced an immunogenic serotype 3 capsular polysaccharide (Gilbert et al. 2000).

Over the past decade, this field of research has been the subject of several reviews. Thus, the aim of this article is not to extensively review the entire literature but to update progress and discuss major new developments and strategies. Ongoing challenges for the future goal of using LAB as mucosal delivery vehicles in human and veterinary medicine is also discussed.

FATE OF LACTIC ACID BACTERIA IN THE HOST AND IMPLICATIONS FOR MUCOSAL DELIVERY

Different species of LAB vary in their capacity to survive passage through the stomach and persist and replicate in the GI tract (Klijn et al. 1995, Vesa et al. 2000). The mucus layer secreted by

goblet cells in the epithelium is a significant physical barrier between microbes and contact with the epithelium. Indeed, studies on human biopsy material indicated that most commensal bacteria were present either in suspension in the lumen or trapped in the mucus (van der Waaij et al. 2005). In the mouse, it was recently shown that the colonic mucus consists of two layers extending 150 μm above the epithelial surface with a similar protein composition (Johansson et al. 2008). Whereas the inner layer is densely packed and devoid of bacteria, the outer layer is less dense and colonized by bacteria (Johansson et al. 2008). In the small intestine, less is known about the composition and extent to which the secreted mucus layer covers the entire epithelium. However, in the small intestine the follicular epithelium covering the mucosal-associated lymphoid tissue of the Peyer's patches (PP) is considered to be more accessible to antigens and bacteria present in the luminal compartment. Here, specialized antigen sampling cells (M cells) in the follicular-associated epithelium (FAE) take up particulate antigens and specific binding proteins (e.g., cholera holotoxin or the pentameric binding domain) by endocytosis and transport them to the underlying immune cells. Dendritic cells (DCs) residing in the dome region of the lymphoid follicles are activated by contact with microbial antigens and then migrate to the draining lymph nodes, where they prime T cell responses. After being primed, naive T and B cells become memory/effector cells and migrate from the gut-associated lymphoid tissue (GALT) efferent lymph vessels to the draining lymph nodes and then via the thoracic duct to peripheral blood to other mucosal effector sites such as the lamina propria (LP). Homing of primed lymphocytes to distal mucosal sites is controlled by the profile of adhesion molecules and chemokines expressed on the endothelial cells of the gut microvasculature and is the basis for the compartmentalization of mucosal immune responses (Hanson 1959). M cell–mediated uptake of bacteria is also likely to occur in the isolated lymphoid follicles associated with the colonic epithelium.

In vitro coculture assays of human ileal tissue and Caco2 cell monolayers with nonpathogenic *Escherichia coli* has shown transcellular uptake of bacteria occurs in the FAE but also at lower levels in small intestinal enterocytes of the normal epithelium. Intestinal bacteria may also be sampled directly at the epithelial surface by laminar propia DCs and macrophages, which can extend protrusions through the epithelial tight junctions to the luminal compartment (Rescigno et al. 2001). This process is stimulated by infection with pathogens such as *Salmonella*, but it is not clear to what extent it contributes to the interaction of LAB with the host immune system. Definitive information on the sampling of LAB at mucosal surfaces is lacking, but uptake into the mucosal-associated lymphoid tissue is likely to be important in the induction of immune responses. This implies that the interaction of different LAB with dendritic cells will play a key role in determining the nature of the immune response.

DCs are the most important professional antigen-presenting cells (APC) and express up to 100-fold more major histocompatibility (MHC) antigen and are more effective at differentiating naive T cells than other APC (Inaba 1997, Levin et al. 1993). Microbial activation of DCs in the LP and PP is mediated via the binding of microbe-associated molecular patterns (MAMPs) to pattern recognition receptors (PRRs) expressed by mucosal dendritic cells. One such family of PRRs comprises the Toll-like receptors (TLRs), which are expressed by a variety of cells of the innate immune system, including immature DCs. Each TLR family member is endowed with the ability to recognize a distinct class of conserved MAMPs. Another class of PRRs are the nucleotide-binding and oligomerization domain (NOD)-like receptors NOD1 and NOD2, which recognize the synthetic peptidoglycan structures meso-diaminopimelic acid (meso-DAP) and muramyl dipeptide (MDP), respectively. The nature and combination of the different signals encountered by DC is known to shape the course of the immune response (Wells et al. 2010a). Several studies have shown that different LAB, and even different strains of the same species, have markedly different effects on DC maturation and cytokine production in vitro (Christensen et al.

2002, Meijerink et al. 2010). Additionally, there is evidence that the immune profile obtained in coculture assays with LAB in vitro (especially for Il-10 and Il-12) can be predictive of their in vivo immunomodulatory activities (Foligne et al. 2007, Kwon et al. 2010). This suggests that LAB strains for vaccine applications could be specifically selected on the basis of their ability to modulate DC function and prime T cells for a Th1, Th2, or mixed Th1/Th2 response.

FACTORS INFLUENCING THE IMMUNOGENICITY OF LAB VACCINES

Different LAB have been investigated as vaccine delivery vehicles using the non-toxic tetanus toxin fragment C (TTFC) as a model antigen including *L. lactis*, which passes only transiently through the GI tract and *L. plantarum*, which can persist in mice for several days (Grangette et al. 2004, Mercenier et al. 2000, Norton et al. 1996, Wells et al. 1996). All LAB vehicles induced protective responses but were not directly comparable because of differences in dosing, antigen expression level, and other methodologies (Grangette et al. 2002, Grangette et al. 2001, Norton et al. 1997, Robinson et al. 1997, Shaw et al. 2000, Wells & Mercenier 2008). The immune response to TTFC upon injection with alum adjuvant is dominated by a T helper 2 (T_H2) response and the production of IgG1 antibody, whereas mucosal delivery of TTFC-expressing *L. plantarum* and *L. lactis* promoted a mixed T_H-cell response (Grangette et al. 2002, Grangette et al. 2001, Norton et al. 1996, Robinson et al. 2004) (**Table 1**). This may in part be due to the use of a mucosal route of vaccination as a more pronounced Th1 response to TTFC is obtained when TTFC-expressing *L. lactis* was administered parenterally (Robinson et al. 2004) (**Table 1**).

Recently, *L. lactis* and *S. gordonii* (a non-food associated LAB) have been compared as vaccine delivery vehicles for a vaccine against *Giardia lamblia* (Lee et al. 2009). Both LAB were engineered to express recombinant *G. lamblia* cyst wall protein 2 (CWP2) on the bacterial cell surface as a fusion to the C terminal half of the M6 molecule. CWP2 was previously shown to reduce cyst excretion in infected animals following mucosal immunization with cholera toxin as an adjuvant (Larocque et al. 2003). Interruption of encystations is an attractive control measure against *Giardia* transmission, as the cysts allow parasite survival in the environment and thus transmission between susceptible hosts. Oral administration using different regimes for both vehicles increased CD4+ T helper and B cells in the mesenteric lymph nodes and PP of immunized mice and elicited an IgA response that was higher in mice immunized with *S. gordonii* than *L. lactis*. In challenge studies, mice vaccinated with the *L. lactis* and *S. gordonii* expressing rCWP2 showed significantly reduced cyst output by 71% and 90%, respectively. Additionally, the *S. gordonii*–vaccinated group shed 65% fewer cysts than the *L. lactis* counterparts. The differences in efficacy were not due to higher expression of rCWP2 in *S. gordonii*, as approximately four times more antigen was produced by the *L. lactis* vaccine strain. Analysis of the T cell responses to the vaccine strains revealed a balanced Th1/Th2 cytokine response to CWP2 delivered using *L. lactis* as reported previously for TTFC antigen (Robinson et al. 2004). In contrast, the *S. gordonii* vaccine strain induced a predominant Il-12 (Th1 cytokine response) to CWP2. Given the established role of Il-12 and CWP2-specific IgA in protection against *Giardia*, it seems likely that these factors were responsible for the superior performance of the *S. gordonii* vaccine. In contrast to *L. lactis*, which passes only transiently through the mouse GI tract, *S. gordonii* can persist for up to 30 days, and this may have contributed to the higher IgA response to CWP2 (Lee & Faubert 2006).

Several LAB vaccine studies have investigated the effect of antigen location (cytoplasmic, secreted, or anchored to the cell wall) on immunogenicity. Nevertheless, it has been difficult to conclude which location of the antigen provided optimal mucosal immunization because of strain differences in the amount of expressed antigen and the fact that a proportion of secreted antigen

may remain cell associated depending on the construct and level of expression (reviewed by Wells & Mercenier 2008). More recently, the immunogenicity of recombinant strains of *L. casei* ATCC 393 either expressing infectious bursal disease virus (IBDV) capsid 2 antigen (VP2) anchored to the cell wall or secreting VP2 into the milieu was compared by oral immunization of mice (Yigang & Yijing 2008). Both strains elicited IgA in the intestinal lavages and serum IgG, which reacted with VP2 and neutralized virus in a plaque-forming assay on confluent cells in vitro. The highest levels of VP2-specific antibody and inhibition of viral plaque formation were obtained with the strain secreting VP2. This was somewhat surprising given that oral immunization with soluble antigens typically leads to oral tolerance (OT) and that a previous LAB vaccine study concluded that secreting the antigen was the least immunogenic (Bermudez-Humaran et al. 2004). One possibility is that a proportion of VP2 produced by the secretion vector remained cell associated and impacted on the immune response.

Expression of the OspA lipoprotein, a protective antigen against *Borrelia burgdorferi*, in *L. plantarum* was shown to elicit a protective antibody response in mice with Lyme disease (del Rio et al. 2008) (**Table 1**). More recently, the influence of lipidation on the immunogenicity of this antigen was investigated by del Rio et al. (2010). The *ospA* gene was mutated to change the cysteine at position 17 in the lipidation motif of OspA to aspartic acid, resulting in lack of lipid attachment. Assays with both forms of OspA showed that lipidation increased the immune responses of dendritic cells to purified OspA, presumably through binding of the diacyl groups to TLR2/6, resulting in immune activation. Higher amounts of cytokines were also induced in human peripheral blood mononuclear cells (PBMC) and DC coculture assays with *L. plantarum* expressing the lipidated form of OspA. Interestingly, recombinant strains of OspA-expressing *L. plantarum* or mutated OspA-expressing *L. plantarum* induced OspA-specific IgA in the bronchoalveolar lavage and stool suspensions as well as IgG1 and IgG2a serum antibodies to OspA. However, higher levels of OspA-specific IgG1 were elicited by the strain expressing the lipidated OspA, reflecting a shift from a Th2 to a Th1 response. These results confirm previous studies showing that the lipidation of OspA is a critical determinant of its immunogenicity (Erdile et al. 1993) and also highlight the potential to use OspA as a fusion partner for other antigens to enhance Th1 responses.

NOVEL STRATEGIES FOR ENHANCING LAB VACCINES

The fact that DCs play a key role in the induction of mucosal immunity to bacterial antigens was recently exploited to potentiate mucosal immune responses to the protective antigen (PA) of *Bacillus anthracis* (Mohamadzadeh et al. 2009). In this study, *L. acidophilus* was engineered to secrete the PA of *B. anthracis* fused to a 12 amino acid peptide (DCpep) that specifically binds to DCs and promotes endocytosis. The targeting peptide was selected from a phage display library screen and was fused to hepatitis C viral antigen and shown to elicit efficient antigen-specific responses without modulating the function of DCs (Mohamadzadeh et al. 2009). The efficiency of the strategy was tested by oral immunization of mice and challenge with a lethal dose of the *B. anthracis* Sterne strain. Oral vaccination with *L. acidophilus* expressing PA-DCpep induced anti-PA neutralizing antibodies and T cell immunity against *B. anthracis* (**Table 1**). The immune responses were comparable to those obtained with the current vaccine comprising recombinant PA and aluminum hydroxide given subcutaneously (s.c.). In comparison, *L. acidophilus* expressing PA fused to a control peptide showed only minor protection, and no protection was seen with *L. acidophilus* carrying the empty vector. The current recombinant PA vaccine is far from ideal, as it is administered in multiple s.c. injections and is reactogenic in some individuals. If the recombinant LAB (rLAB) vaccine for *B. anthracis* is further optimized and engineered to contain the transgene by an approach that is acceptable to the regulatory authorities, it

Table 1 Protection studies with lactic acid bacteria vaccines

Vaccine target	Vehicle	Antigen (mode)	Model (route)	Immune responses[a]	Protection model (outcome)	References
Helicobacter pylori	LP, LP *alr*	Urease B (cyt)	Mouse i.g.	Serum Ab	Colonization level (partial protection)	Corthesy et al. 2005
Helicobacter pylori	LL	Urease B (cyt)	Mouse i.g.	Not significant	Colonization level (no protection)	Lee 2003
Tetanus	LL	TTFC (cyt)	Mouse i.g., i.n., s.c.	Serum Ab, fecal IgA T cells, ELISPOT	Survival after tetanus toxin challenge (protection)	Norton et al. 1996, Robinson et al. 2004, Robinson et al. 1997, Wells et al. 1993
Tetanus	LP, LL, LP *alr*	TTFC (cyto)	Mouse i.g., i.n., intravaginal	Serum Ab, BALF, T cells, neutralizing Ab	Survival after tetanus toxin challenge (protection)	Grangette et al. 2002, Grangette et al. 2001, Grangette et al. 2004
Streptococcus pneumoniae	LL	PspA	Mouse i.n.	Serum Ab, BALF Ab	Infectious lethal challenge i.p. and i.n. (increased survival)	Hanniffy et al. 2007
Streptococcus pneumoniae	LP, LH	PsaA	Mouse i.n.	Ab in serum BALF, nasal wash	Nasal colonization (reduction in pneumococci)	Oliveira et al. 2006
Streptococcus pyogenes	LL	CRR of M protein serotype 6 (cwa)	Mouse i.n., s.c	Salivary IgA, serum Ab	Pharyngeal infection (i.n. route protective)	Mannam et al. 2004
HIV	LL	V2-V4 loop of gp120 (cwa)	Mouse i.g. with CT adjuvant	Serum Ab, fecal Ab, ICCS, tetramer assay, ELISPOT	Intraperitoneal challenge with HIV Env expressing vaccinia virus (viral load reduced)	Xin et al. 2003
Erysipelothrix rhusiopathiae	LL	SpaA (cwa)	Mouse i.n.	Serum Ab, fecal IgA	Challenge with *E. rhusiopathiae* (protection from death)	Cheun et al. 2004
Enterotoxigenic *Escherichia coli*	LA	K99 fimbriae	Pig intestinal brush border ex vivo	N/A	Inhibition of K99+ *E.coli* adhesion in porcine intestinal brush border	Chu et al. 2005
SARS-associated coronavirus	LC	Spike antigen segments (cm)	Mouse i.g., i.n.	Serum Ab, mucosal IgA	Viral neutralizing antibody elicited	Lee et al. 2006

Rotavirus	LL	VP7 (cyt, cwa, sec)	Mouse i.g.	Serum Ab	Virus neutralization assay (neutralizing Ab demonstrated for VP7 (cwa) vaccine)	Perez et al. 2005
IBDV	LL	VP2, VP3 (cyt, cwa, sec)	Chickens oral	No detectable immune response to antigens	Not performed, as no immune response detectable	Dieye et al. 2003
Group B *Streptococcus*	LL	Pilus (Island 1) (cwa)	Mouse s.c., i.p., i.n.	Serum Ab, Ab in nasal and vaginal washes	Survival of offspring from vaccinated mothers after infectious challenge	Buccato et al. 2006
Brucella abortus	LL	L7/L12 (cyt)	Mouse i.g.	Fecal IgA	Partial protection against i.p. inoculation of virulent *B. abortus*	Pontes et al. 2003
HPV-16-induced tumors	LL	E7 (cwa) plus IL-12 (sec)	Mouse i.g.	CTL, ELISPOT	Protection demonstrated against injection of E7-expressing tumor cell line	Bermudez-Humaran et al. 2005
HPV-16-induced tumors	LC	E7	Mouse i.g.	Serum Ab, mucosal IgA ELISPOT	Protection demonstrated against injection of E7-expressing tumor cell line	Poo et al. 2006
Plasmodium yoelii	LL	MSP-1 (cyt)	Mouse i.g.		Challenge with *P. yoelii* parasites (reduced parasitemia)	Zhang et al. 2005
Bacillus anthracis	LA	PA fused to DCpep	Mouse i.g.	Serum Ab, IgA staining of cells, PA neutralizing Ab, T cell responses	Increased mouse survival after challenge with *B. anthracis*. Protective immunity was equivalent to injected PA plus alum.	Mohamadzadeh et al. 2009
Enteropathogenic *E. coli* (EPEC)	LC	Intimin β	Mouse i.n.	IgA in nasal washes, serum Ab	Induction of serum antibodies that inhibit binding of EPEC to epithelial cells	Ferreira et al. 2008
Giardia lamblia	LL, SG	Cyst wall protein-2 (CWP-2)	Mouse i.g.	ELISA for intestinal IgG & IgA, cytokine ELISPOT mucosal leukocyte cultivation and analysis	Reduction in cyst output in infected mice. SG was superior to LL as a CWP-2 vaccine vehicle	Lee et al. 2009

(*Continued*)

Table 1 (*Continued*)

Vaccine target	Vehicle	Antigen (mode)	Model (route)	Immune responses[a]	Protection model (outcome)	References
Yersinia pseudotuberculosis	LL	Low calcium response antigen (LcrV)	Mouse i.n.	Serum Ab, ELISA of IgA in intestinal & BALF, cytokine assays on splenocytes	Protection against oral & systemic challenges with *Yersina*	Daniel et al. 2009
Enterotoxigenic *E.coli* (ETEC)	LC	F41 fimbrial antigen	Mouse i.g.	IgA in intestinal and BALF by ELISA, serum IgG	Post-infectious survival rates of mice after challenge with ETEC. LC vaccination increased survival	Liu et al. 2009
Borrelia burgdorferi	LP	OspA	Mouse i.g.	Fecal IgA, IgG	LP showed complete protection from challenge with *B. burgdorferi* infected ticks.	del Rio et al. 2008
Streptococcus pneumoniae	LC	PspA	Mouse i.n.	Serum IgG, IgA in nasal washes and saliva	Intraperitoneal challenge with virulent pneumococci. Increased survival shown for LC expressing PspA	Campos et al. 2008
S. pneumoniae	LC	PspA, PspC	Mouse i.n.	Serum and vaginal Ab, ELISPOT and ELISA for IFN-γ and Il-17 secretion in lung and spleen cells	Respiratory challenge with virulent pneumococci. Increased survival seen with LC expressing PspA and rPspA without adjuvant	Ferreira et al. 2009
Listeria monocytogenes	LL	Listeriolysin O	Mouse i.p., i.g	LLO-specific T cells by ELISPOT, serum Ab	Challenge with luminescent *Listeria*. Organ luminescence and *Listeria* counts showed partial protection with LL vaccine strain	Bahey-El-Din et al. 2008

[a]Responses detected using any of the indicated vaccination routes.

LP, *Lactobacillus plantarum*; LL, *Lactococcus lactis*; LC, *Lactobacillus casei*, LA, *Lactobacillus acidophilus*; LH, *Lactobacillus helveti*cus; SG, *Streptococcus gordonii*; cyt, cytoplasmic; cwa, cell wall–associated; sec, secreted; i.g., intragastric; i.n., intranasal; s.c., subcutaneous; Ab, antibody; BALF, bronchoalveolar lavage fluid; IgG, immunoglobulin G; IgA, immunoglobulin A; PA, protective antigen; TTFC, tetanus fragment C; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C; CT, cholera toxin; Ab, antibodies; CTL, cytotoxic lymphocytes; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot assay; LLO, listeriolysin; IFN-γ, interferon gamma; ICCS, intracellular cytokine staining assay; CRR, C-repeat region; IBDV, infectious bursal disease virus (IBDV); VP2, virus capsid 2 antigen; VP7, virus outer-layer protein seven; MSP-1, merozoite specific protein 1; OspA, outer-membrane surface protein A; SpaA, surface protective antigen A.

would have considerable potential to be developed as a mucosal vaccine against this deadly pathogen.

Another targeting strategy employing the *E. coli* heat labile toxin B (LTB) subunit protein was recently evaluated as an approach to enhance immune responses to *L. casei* expressing a recombinant porcine rotavirus antigen VP4 (Qiao et al. 2009). Like *Vibrio cholera* toxin, LTB has been shown to be a mucosal adjuvant by virtue of the ADP-ribosylating activity of the A subunit and the GM1-gangliside binding activity of the pentameric B subunit. However, the holotoxins are too toxic for use in humans. As GM-1 is present on the follicular epithelium covering the PP, the nontoxic pentameric B subunits of both toxins have been explored as targeting molecules for recombinant and conjugated vaccine antigens (Yamamoto et al. 2001). *L. casei* expressing the major protective antigen VP4 of porcine rotavirus alone or as a fusion to VP4 were used to immunize mice. Both strains elicited a VP4-specific serum IgG response and VP4-specific IgA in the ophthalmic and vaginal washes after oral immunization (**Table 1**). The IgA titres were significantly higher in the mice immunized with the *L. casei* expressing recombinant VP4-LTB than in *L. casei* expressing only VP4 (Qiao et al. 2009). These authors concluded that LTB served as a mucosal adjuvant, but it is not clear whether the two vaccine strains expressed similar levels of amounts of antigen or whether the VP4-LTB protein could form pentamers on the cell surface of *L. casei*, which are capable of binding to GM-1.

Bacterial flagellin was recently explored as a fusion partner for vaccine antigens expressed in *L. casei* (Kajikawa et al. 2007, 2010). Flagellins can be protective antigens themselves but when expressed as a fusion protein with other antigens can also act as an adjuvant. This is most likely related to the ability of flagellin to bind to the innate TLR5 receptor and trigger host inflammatory responses. The SipC antigen of *Salmonella enterica* was expressed in *L. casei* alone and also as a fusion protein linked to the N or C terminus of flagellin in the pLP401 vector, which provided sequences for secretion and a cell wall anchoring of the recombinant protein. These strains all elicited similar responses to SipC, following intraperitoneal injection indicating that flagellin did not adjuvant the antibody responses to this antigen. The antibody titres to flagellin appeared to be influenced by the nature of the fusion protein, and highest titres were obtained with the strain that expressed SipC as a C-terminal fusion to flagellin. Further work is needed to conclusively determine whether flagellin could adjuvant immune responses to other antigens expressed in LAB, including an investigation into mucosal routes of administration and different modes of expression.

Another interesting development has exploited the recent discovery of pili in Gram-positive bacteria as antigen display systems (Quigley et al. 2010). As a proof of principle the maltose binding protein (MBP) of *E. coli* was fused to the C terminus of the tip protein from the T3 pilus of *Streptococcus pyogenes* and expressed in *L. lactis*. Intranasal immunization with this strain elicited mucosal IgA and serum IgG responses to MBP. This method of antigen presentation remains to be compared directly with other modes of expression (e.g., intracellular, cell wall anchored), but it seems to be a promising strategy for presentation of vaccine polypeptides in LAB.

Following the first demonstration that *L. lactis* could secrete biologically active Il-2 and Il-6 and stimulate mucosal and systemic responses to the model antigen TTFC (Steidler et al. 1995, 1998), several studies have sought to use the coexpression of cytokines to modulate LAB vaccine responses. Coadministration of an Il-12-secreting strain of *L. lactis* with another strain of *L. lactis* expressing a cell wall–anchored form of the E7 antigen from human papilloma virus (HPV)-16 increased protection in a mouse cancer model (Bermudez-Humaran et al. 2005). Recently, the adjuvant effects of *L. casei* secreting murine Il-1β were investigated in combination with a heat-killed *Salmonella enterica* serovar Entertidis (SE) vaccine. Biologically active murine IL-1β was secreted effectively at levels of up to 1 ug ml^{-1} in the culture supernatant. Intragastric immunization with LL-Il1-β elevated both serum IgG and mucosal IgA responses to the SE vaccine (Kajikawa et al. 2010).

The chemokines Mig and IP-10 have also been investigated as potential vaccine adjuvants owing to their pronounced chemotactic activities on mononuclear cells such as T cells, natural killer (NK) cells, and monocytes (Cortes-Perez et al. 2008). Intranasal administration of *L. lactis* producing a secreted Mig-IP-10 fusion protein and a cell wall–anchored form of the E7 antigen from HPV or *L. lactis* expressing E7 and control groups was used to investigate the potential adjuvant effect of Mig-IP-10. The humoral responses to E7 antigen were substantially higher in mice immunized with the strain expressing the Mig-IP-10 protein, suggesting that Mig-IP10 does indeed have immunostimulatory properties in vivo (Cortes-Perez et al. 2008) (**Table 1**).

PROTECTION STUDIES WITH LACTIC ACID BACTERIA VACCINES

Since the field was last extensively reviewed (Wells & Mercenier 2008), there have been several new publications describing the expression of different candidate vaccine antigens in LAB (Adachi et al. 2010, Cortes-Perez et al. 2009, Kim et al. 2009, Li et al. 2010, Liu et al. 2010, Qiao et al. 2009, Tang & Li 2009), some of which have been evaluated in protection models in vivo (Campos et al. 2008; Daniel et al. 2009; del Rio et al. 2008; Ferreira et al. 2008, 2009; Liu et al. 2009) (**Table 1**). The future development and implementation of LAB vaccines will depend on several factors, including relative cost, acceptability as contained genetically modified organisms (GMOs), and efficacy. A necessary step is to show that the subunit LAB vaccines confer an advantage over traditional routes of immunization and sufficient efficacy in protection models (e.g., compared to injected vaccines). To date, there are only a few LAB vaccine protection studies that directly compare immunogenicity and protection with administration of the antigen together with adjuvant (Corthesy et al. 2005, Hanniffy et al. 2007, Mohamadzadeh et al. 2009). Partial protection against *Helicobacter felis* was demonstrated in mice by the use of recombinant *L. plantarum* NCIMB8826 strains producing the urease B antigen, but this was not as effective as vaccination with the antigen plus cholera toxin as an adjuvant (Corthesy et al. 2005). Protective vaccination against *B. anthracis* was obtained by vaccination with *L. acidophilus* expressing the PA fused to a DC-targeting peptide (see above). The protective immunity induced by oral vaccination with rLAB was equivalent to that obtained by injection of PA with alum (Mohamadzadeh et al. 2009). Intranasal administration of *L. lactis* expressing pneumococcal surface protein A (PspA) afforded better protection against respiratory challenge with virulent pneumococci than intra-nasal (i.n.) PspA or PspA injected with alum Hanniffy et al. 2007. This important advance demonstrated that a LAB vaccine could be more effective than an injected vaccine in an in vivo challenge model. The higher protection afforded by *L. lactis* was attributed to a shift toward a T_H1 response compared to the injected antigen. Additionally, the lactococcal vaccine afforded protection on a par with that obtained with the injected vaccine in a sepsis model of pneumocccal disease. Decreased colonization of *S. pneumoniae* has also been observed in mice following nasal inoculation of different LAB expressing pneumococcal PspA (Oliveira et al. 2006). More recently, PspA-expressing *L. casei* was shown to induce cross-protective antibodies to both clade 1 and clade 2 variants of PspA and confer protection in a sepsis challenge model using a heterologous strain of pneumococcus (Campos et al. 2008). An independent study recently compared immune responses and protection from infectious challenge to different *L. casei* strains expressing the candidate vaccine antigens PspA and PspC, both of which have been shown to be protective protein antigens in challenge models. Immunization with the *L. casei* vaccine strains and PspC protein without adjuvant failed to elicit antigen-specific serum antibodies by the intranasal route. Via the s.c. route of immunization, only the purified protein elicited a specific humoral response. In contrast, PspA was found to be more immunogenic than PspC and i.n. immunization with purified PspA (clade5) and PspA5-expressing *L. casei* elicited anti-PspA serum antibodies and conferred protection against an i.n. challenge with

virulent *S. pneumonia* (Ferreira et al. 2009). Humoral responses and mean survival time of challenged mice were higher in the group immunized with purified PspA5 without adjuvant. Cellular responses to the vaccines were also measured, and the highest levels of protection were characterized by increased levels of Il-17 and IFN-γ by lung and spleen cells, respectively, as well as low levels of TNF-β in the respiratory tract.

L. plantarum expressing OspA, a protective antigen against *B. burgdorferi*, in animals and humans has been evaluated as an experimental vaccine against Lyme disease in mice (del Rio et al. 2008). The mechanism of protection is somewhat unconventional for a vaccine, as it is aimed at blocking *B. burgdorferi* ticks in the midgut of the tick vector. OspA-expressing *L. plantarum* strains but not the control strain carrying the vector alone elicited mucosal sIgA and systemic IgG humoral responses following i.g. immunization. Furthermore, the OspA-expressing *L. plantarum* strains protected mice from challenge with *B. burgdorferi* infected ticks, which are the natural vectors of infection. One month after challenge, the immunized mice were shown to be free of *B. burgdorferi* by culture, polymerase chain reaction (PCR), and immunoblotting with serum raised against whole extracts of the pathogen. Interestingly, this study also showed that a mutant OspA lacking a potential autoantigenic epitope (Chen et al. 1999) was protective using *L. plantarum* as a delivery vehicle. This strain of *L. plantarum* did not colonize the gut, and higher numbers of *L. plantarum* (4×10^{10}) were used for intra-gastric (i.g.) vaccination than in many previous LAB vaccine studies. Furthermore the priming doses were given twice daily on days 1–4 and 8–11, and the booster immunizations twice on days 30–33 and days 52–55. The longer period of immunization and more frequent dosing may have contributed to the impressive protective capacity of the vaccine in mice.

DNA VACCINE DELIVERY

A recent development in the use of LAB as delivery vehicles has been in the field of DNA vaccination. The advantage of DNA vaccines lies in their ability to induce potent cellular immune responses in addition to antibodies and the flexibility to express multiple antigens or epitopes using one DNA vector. For viral antigens, the correct posttranslational modifications (e.g., glycosylation) should be carried out by the host cell machinery. Despite the successful use of DNA vaccination in small animals, its translation to primates, humans, and other large animals has been beset with problems (Jechlinger 2006).

Delivery of DNA into mammalian cells was demonstrated using native lactococci and a plasmid carrying the bovine β-lactoglobulin (BLG) gene under the control of the viral promoter P_{cmv}, which is not functional in *L. lactis* (Guimaraes et al. 2006). Expression of BLG was detected by PCR in Caco2 cells after incubation with *L. lactis* carrying the DNA vaccine vector expression plasmid, but not after incubation with the purified recombinant plasmid alone, or when the plasmid was mixed together with *L. lactis*. Although the efficiency of delivery was low this study showed clear potential to further optimize LAB as DNA vaccine delivery vehicles. Since then, recombinant *L. lactis* expressing internalin A, a cell wall–anchored protein and major invasin of *L. monocytogenes*, was shown to be internalized by epithelial Caco2 cells in vitro and enterocytes in vivo after administration to guinea pigs (Guimaraes et al. 2005). In vivo, the invasiveness of the internalin expressing strain was about 100-fold higher than for the *L. lactis* control strain. The ability of the strains to deliver a vaccine vector expressing green-fluorescent protein (GFP) under the control of the P_{cmv} promoter was also assessed in vitro. The bacterial strains were added at a ratio of 1000:1 bacteria per Caco2 cell, of which about 1% of the internalin-expressing lactococci were internalized by Caco2 cells (Guimaraes et al. 2005). Only 1.2% of the Caco2 cells expressed eGFP indicating that only some of the cells internalized several bacteria or that internalization does not always lead

to plasmid transfer and expression. Recently, work by the same group showed that CFSE-labeled *L. lactis* were in fact not evenly distributed among the Caco2 cells and were predominantly located at the periphery of the cell clusters (Innocentin et al. 2009). Similar results were obtained with *L. lactis* expressing the fibronectin-binding protein A (FnBPA) of *Staphylococcus aureus* (Innocentin et al. 2009). These results are compatible with the basal lateral membrane localization of the host receptors for these bacterial invasion proteins. E-caderin, the receptor for internalin A, is not usually exposed at the surface of the intestinal epithelium, but it has been suggested that *Listeria* invade at the tip of the villi where apoptosis of enterocytes temporarily exposes the basolateral membrane and thus E-cadherin to the lumen.

One approach that may increase the efficiency of DNA vaccine delivery involves the use of listeriolysin (LLO) from pathogenic *L. monocytogenes*. Upon internalization into an endosome or phagosome, the acidification process activates LLO, allowing it to form pores in the membrane and permit escape of the bacteria. Although not yet utilized for DNA vaccine delivery, a strain of *L. lactis* expressing LLO has been tested for its ability to protect mice against live intraperitoneal challenge with *L. monocytogenes* (**Table 1**). The strain secreting LLO at the highest levels induced CD8+ T cell responses, indicating that LLO was presented via the cytosolic MHC class I pathway. This strain conferred protection against a *Listeria* challenge but only when administered by the intraperitoneal (i.p.) route but not the i.g. route (Bahey-El-Din et al. 2008). When the expression of LLO was combined with a truncated form of the *Listeria* P60 antigen, no significant improvement protection was observed over the *L. lactis* strain expressing only LLO. The expression of tP60 was relatively low and possibly insufficient for access to the cytoplasm (Bahey-El-Din et al. 2010).

An experimental DNA vaccine using *L. acidophilus* as a carrier for use against foot-and-mouth-disease virus was recently described (Li et al. 2007). Significant immune responses to the vaccine antigen were only measured or reported using the injected routes of administration, although it was indicated that mucosal administration could prime a specific immune response (Li et al. 2007). Clearly, there is some way to go before LAB can effectively deliver DNA vaccines via the oral route, but there is clear potential to optimize targeting strategies and combine this with LLO expression to facilitate release of the DNA vaccine into the cytoplasm. The next step will then be to demonstrate their immunogenicity and efficacy in animal models.

THERAPEUTIC APPLICATIONS IN INFLAMMATORY BOWEL DISEASE

Many species of LAB are members of the intestinal microbiota of humans and animals, and as such represent good vehicles for delivery of therapeutic biologicals to the GI tract. The mucosal immune system is faced with the difficult task of balancing opposing immune functions: immunological tolerance to harmless food or bacterial antigens and immunity to pathogenic organisms. Several important human intestinal disorders are associated with loss of homeostasis to harmless antigens, including food allergy, IBD, celiac disease, and autoimmune diseases. It is not surprising that the mechanisms supporting homeostasis of tolerance and immunity remain a highly active area of investigation (Wells et al. 2010b). Genetically modified LAB have the potential to produce and secrete therapeutic proteins in situ at different sites within the intestinal tract, thereby increasing availability of the therapeutic target cells. Consequently, the therapeutic dose is expected to be lower if delivered locally in the mucosa, thus avoiding the need for higher systemic concentrations and the risk of side effects. Furthermore, the production of biologicals is often difficult and costly.

The first therapeutic application of rLAB was based on the secretion of the inflammatory cytokine Il-10 by *L. lactis* and treatment of experimental colitis in mice as a model for IBD in humans (Steidler et al. 1998). IBD is a chronic inflammatory disorder with an average yearly

incidence of 10–15 persons per 100,000 individuals in Western countries. The two major forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). The symptoms include bloody diarrhea or constipation, abdominal pain, fever, and weight loss. There is no cure, and therapy is focused on alleviation of the active disease and the accompanying symptoms.

In mice, the daily administration of Il-10-secreting *L. lactis* caused a 50% reduction in colitis induced by dextran sulfate sodium (DSS) at a dose that was 10,000-fold lower than that systemically administered. Treatment of of Il-$10^{-/-}$ Sv/Ev mice that spontaneously develop a severe colitis, with the Il-10-secreting *L. lactis* also prevented the onset of colitis (Steidler et al. 1998). This beneficial effect was dependent on secretion of Il-10 in situ by live lactococci. An environmentally contained version of the hIL-10-secreting strain (see section on Environmental Containment below) has also been evaluated in an open label Phase I trial in CD patients (Braat et al. 2006). The strain was formulated in capsules to optimize survival during passage through the stomach. During the trial, 8 out of 10 patients with moderate to severe disease showed significant clinical improvement, and five went into complete remission. In addition, the containment strategy was validated in humans, and no serious adverse effects were reported. A large Phase 2 trial is reported to be underway to further evaluate the safety, tolerability, and efficacy of the hIl-10-secreting *L. lactis* in UC patients (Rottiers et al. 2009).

In addition, there is potential to use the secretion of molecules, such as trefoil factors (TFF) or TNF blocking antibodies, by *L. lactis* to treat IBD. TFFs are a family of small peptides that promote mucosal protection and repair through the use of multiple mechanisms and also have effects on the immune system. TFFs are highly stable to acid denaturation and proteolytic degradation but are not effective as therapeutic agents when administered orally because they adhere to the mucus that is continuously removed from the intestine by peristalsis. However, i.g. administration of TFF-secreting *L. lactis* during or after DSS induction of colitis in mice resulted in the reduction of several disease parameters, including body weight and histological and inflammatory markers. Even rectal administration of 2,500-fold higher doses of TFF was not as effective as TFF-secreting *L. lactis*, suggesting that *L. lactis* is able to deliver TFF peptides in close proximity to the colonic epithelium (Vandenbroucke et al. 2004).

Monoclonal antibodies such as infliximab, which neutralize the proinflammatory cytokine TNF, are also being used as part of the therapeutic armamentarium of IBD. However, treatment requires periodic infusions of relatively large amounts of purified antibody. Other biologics include monoclonal antibodies that block leukocyte adhesion or T cell signaling via the Il-6 receptor as well as Il-12/23 cytokine blocking antibodies. Recently, *L. lactis* was engineered to secrete an anti-TNF single-domain antibody fragment derived from heavy chain camelid antibodies and shown to be effective in the treatment of DSS-induced colitis and established colitis in the Il-$10^{-/-}$ mouse model (Rottiers et al. 2009). In this study, anti-TNF antibodies were shown to be associated with the surface of cells of the lamina propia, where leukocyte infiltration was evident but was not measured in systemic circulation. This suggests that oral delivery would prevent the systemic side effects associated with injection of the antibodies in humans.

Another new strategy for treatment of colitis was reported based on secretion of low calcium response protein (LcrV), a protein produced by pathogenic *Yersinia*, to evade the host's immune response (Foligne et al. 2007). This protein induces and stimulates Il-10 production in the host mucosal tissues. The protective and therapeutic potential of LcrV-secreting *L. lactis* was demonstrated in the trinitrobenzene sulfonic (TNBS) acid and DSS mouse models of colitis. In the TNBS-induced colitis model the protective effect of *L. lactis* secreting LcrV was about 50% based on the macroscopic lesion score (Wallace score) and as efficient as the Il-10 secreting strain that was previously reported (Steidler et al. 2000). In summary, there is considerable potential for mucosal therapy of IBD in humans using rLAB.

PROPHYLAXIS AND THERAPY OF ALLERGIC DISEASE

Allergy is defined as a hypersensitivity reaction mediated by specific antibody-mediated or cell-mediated immunologic mechanisms, the clinical manifestation of which is called allergic disease. The progression of infant allergy to atopic diseases such as atopic eczema, allergic rhinoconjunctivitis, and ultimately asthma is becoming increasingly common and is now referred to as the pediatric allergic march. This is contributing to the so-called epidemic of allergic or atopic diseases that affect up to 30% of the European and U.S. population and are responsible for a substantial healthcare burden on society (Holgate 1999, Kalliomaki et al. 2010).

Recently, i.g. administration of Il-10-secreting *L. lactis* (described above) for three consecutive days before sensitization to BLG was shown to promote OT in young mice (Frossard et al. 2007). Administration of Il-10-secreting *L. lactis* reduced antigen-induced anaphylaxis and almost completely inhibited the production of IgE and IgG1 antibodies to BLG. Interestingly, these protective effects were partly attributable to *L. lactis* itself, as the wild-type strain also diminished the levels of BLG-specific IgE and IgG1. Moreover, the results of this study suggested that the recombinant *L. lactis* strain increased Il-10 levels in the mucosa and promoted the development of antigen-specific sIgA. In addition, the immunomodulatory effects of lactococcal-delivered Il-12 have been investigated in mouse models of OVA-induced asthma and BLG food hypersensitivity (Bermudez-Humaran et al. 2005, Wu et al. 2006). Cytokine-secreting LAB could be considered as a strategy to prevent food allergy, but the possibility of unexpected side effects of cytokines on the immune system is likely to be one of the safety concerns.

Mucosal delivery of allergen-expressing LAB have also been proposed for the immunotherapy of type I allergies. The rationale is based on the finding that some strains of LAB have been shown to modulate T-cell responses to an expressed or coadministered antigen towards a T_H1-type immune response (Chatel et al. 2003, Kruisselbrink et al. 2001, Murosaki et al. 1998, Repa et al. 2003), and the concept that mucosal vaccination against type I allergy may offer some advantages over the subcutaneous route for desensitization (Novak et al. 2004). However, the studies published to date have been focused on the prevention of allergic sensitization in mouse models. For example, oral pretreatment of mice with *L. lactis* strains producing the cow's milk allergen BLG was shown to cause a shift towards a Th1 immune response and decrease the levels of BLG-specific IgE (Adel-Patient et al. 2005). The most effective strains were those producing the highest amounts of BLG.

With respect to aero-allergens, oral administration of dust mite allergen Der p1–expressing *L. plantarum* and Der p5–expressing *L. acidophilus* were both shown to reduce local allergen-induced airway inflammation, hyperreactivity, and allergen-specific IgE production (Charng et al. 2006, Kruisselbrink et al. 2001). The modulation of allergic immune responses to the birch pollen allergen Bet v1 by rLAB has also been recently described (Daniel et al. 2007). Intranasalvaccination with *L. lactis* and *L. plantarum* strains producing substantial amounts of the Bet v1 allergen in two different cellular locations (intracellular, extracellular) enhanced allergen-specific mucosal IgA levels and induced a shift toward a Th1 response in a prophylactic mouse model of type I allergy to birch pollen. Via the intragastric route only recombinant *L. plantarum* was effective (Daniel et al. 2006). The *L. lactis* strain produced about fourfold less Bet v1 than the *L. plantarum* strains, so this may account for the lower efficacy of this strain. This is in agreement with previous observations that the reduction of BLG-specific IgE was most effective using *L. lactis* strains that produced the highest amount of allergen (Adel-Patient et al. 2005). However, differences in intrinsic immunomodulating capacities, gut persistence, or both, of these two strains cannot be ruled out as contributing factors.

The induction of OT to food antigens and autoantigens is an alternative approach to preventing allergic and autoimmune diseases, but several clinical trials attempting to induce OT have failed

(Faria & Weiner 2005, Kraus & Mayer 2005, Weiner 2004). In experimental models, high doses of antigen induced OT by clonal deletion or anergy of T cells recognizing the antigen, whereas low antigen doses led to antigen-specific regulatory T cells (Tregs) (Friedman & Weiner 1994, Yoshida et al. 1997). The capacity of chicken ovalbumin (OVA)-secreting *L. lactis* (LL-OVA) has been evaluated for its capacity to induce OT in transgenic DO11.10 mice, which express an ova-specific T cell receptor on CD4+ T cells (Huibregtse et al. 2007). The doses of OVA secreted in the gut by repeated feeding of *L. lactis* (up to 10 μg) were considerably lower than that typically used for effective low tolerance induction (5 mg) but nevertheless reduced delayed-type hypersensitivity (DTH) responses to OVA. Interestingly, *L. lactis* carrying the empty vector also suppressed ova-specific DTH responses but to a lesser extent than LL-OVA. The suppressive effect was associated with a TGF-β-dependent decrease in the proliferation of OVA-specific splenic T cells. Furthermore, the LL-OVA increased the Foxp3 and CTLA-4 positive cells in the CD4+ CD25-T cell population from 0.045% (control mice) to 4.11%, indicating that it induced Tregs. In contrast, Tregs induction was not detected in the mice fed ovalbumin (1 μg) or the *L. lactis* control strain. This raises the question: By what mechanism did the *L. lactis* control and ovalbumin-fed mice suppress the OVA-specific DTH response? Additionally, it would be interesting to know whether administration of *L. lactis* with low doses of soluble OVA also induces OT, as it may obviate the need to use GMOs.

The approach described above was recently used to investigate the induction of antigen-specific tolerance in an established genotypic celiac disease mouse model (Huibregtse et al. 2009). Celiac disease is caused by loss of tolerance to ingested gliadin and is associated with HLA-DQ2- or HLA-DQ8-restricted T cell responses to specific antigenic epitopes of gliadin. A transgenic NOD mouse expressing the human DQ8 MHC II gene (NOD ABo DQ8) develops autoimmune disease pathology in response to gliadin sensitization (Marietta et al. 2004). *L. lactis* secreting a modified epitope of DQ8 (LL-DQ8) mimicking the deaminated immunodominant epitope of β-gliadin (DQ8d) was administered i.g. on days 1–10 to mice presensitized with the same immunodominant gliadin peptide. As controls, *L. lactis* carrying the empty vector (LL-pT1Nx) and the inoculation buffer alone were administered to different groups of mice. The LL-DQ8 strain effectively suppressed the DTH response to DQ8 compared to the buffer control. Furthermore, treatment with LL-DQ8 also decreased the proliferative capacity of inguinal lymph nodes cells and lamina propria cells. LL-pTNX1, but the not the inoculation buffer control, somewhat reduced DTH response and the cellular proliferative response, but this was smaller than the effects measured in the LL-DQ8-treated mice. In agreement with previous studies using LL-OVA, the mechanism was attributed to an upregulation of Foxp3 Tregs secreting IL-10 and TGF-β, which were detected in the spleen and mucosal tissues of the LL-DQ8-treated mice. Taken together, these results indicate that in the future it might be possible to use rLAB to induce antigen-specific OT in humans as a therapy for autoimmune and allergic diseases.

ANTIINFECTIVE STRATEGIES

Over the past seven years, antiinfective agents have been expressed and tested in LAB with the ultimate aim of using recombinant strains to prevent infection by viral, fungal, or bacterial pathogens at the mucosal surfaces. Several approaches have utilized single chain variable fragments (ScFv) making up variable regions of the heavy and light chains of an immunoglobulin linked together via a linker peptide to neutralize (or immobilize) virions or bacteria before they reach a receptor site on a host cell. One striking example of this therapeutic approach is the protection against experimental vaginitis in rats using *S. gordonii* expressing a secreted or surface-displayed microbiocidal ScFv antibody against *Candida albicans* (Beninati et al. 2000). *Lactobacillus zeae* displaying

a ScFv form of an antibody (Guy 13) directed against the major adhesion molecule of *Streptococcus* mutants was shown to protect against colonization on tooth enamel and dental caries by agglutination and clearance of this bacterium in the mouth (Kruger et al. 2002). In this study, the bacteria were administered daily, but a future aim would be to use a strain that persisted and continuously produced antibodies.

The intercellular adhesion molecule 1 (ICAM-1) receptor is involved in the binding of monocytes and activated CD4+ T lymphocytes to the epithelium, and antibody blocking of this receptor on the cervical epithelium would prevent cell-associated HIV-1 transmission. The expression of a ScFv specific for ICAM-1 in *L. casei* was shown to block monocyte-associated HIV-1 transmission across a cervical epithelial monolayer in vitro (Chancey et al. 2006). Other strategies to block HIV transmission at the mucosa include secretion of microbiocides, peptide inhibitors of viral-cell fusion, and receptors used by HIV to enter cells. For example, the two linked extracellular domains of the CD4 receptor (2D CD4) that binds HIV gp120 envelope protein have been successfully secreted from a vaginal colonizing strain of *Lactobacillus jensenii* at concentrations ranging from 100 ng ml^{-1} to 1 μg ml^{-1} in laboratory culture supernatants (Chang et al. 2003). Furthermore, the secreted 2D CD4 was able to inhibit HIV infection of cultured cells in a dose-dependent manner. Binding of gp120 to CD4 can also be inhibited by the high-affinity interaction of the microbicidal cyanovirin-N (CV-N) protein with the high-mannose structures present on gp120 (Boyd et al. 1997). A number of LAB have been used to express CV-N, including *S. gordonii* (Giomarelli et al. 2002), *L. lactis*, and *L. plantarum* (Pusch et al. 2005) and have been shown to neutralize the infectivity of both laboratory and primary isolates of HIV-1 in vitro (Pusch et al. 2005). More recently, a natural vaginal strain of *L. jensenii* was engineered to express CV-N from a stably integrated expression cassette recombined into the genome (Liu et al. 2006). This strain was capable of colonizing the vagina of mice and producing CV-N in situ, but no protection studies with this strain have yet been published. A different approach to inhibit cell-free HIV infection uses peptide-fusion inhibitors derived from the C-terminal heptad repeat of HIV gp41, as exemplified recently by the work of Rao et al. (2005) using the probiotic *E. coli* Nissle strain as a delivery vehicle. In a similar manner, lactobacilli expressing a HIV-1 fusion-inhibitor peptide have been experimentally evaluated as a potential bioshield (Pusch et al. 2006).

For this strategy to be successful, the colonizing LAB must be able to compete with the resident microbiota and secrete sufficient quantities of the inhibitory proteins to block infection in vivo. Some naturally occurring strains of *Lactobacillus* spp. that were introduced into the vagina to prevent vaginosis have been reported to persist for weeks or even months (Falagas et al. 2007). It is also encouraging to consider that cervico-vaginal transmission of HIV from men to women is an inefficient process (Chakraborty et al. 2001). Nevertheless, the lack of a containment system and thus the potential spread of the genetic trait will be a concern for such an approach in humans.

ENVIRONMENTAL CONTAINMENT

To address safety concerns over the use of Il-10-secreting *L. lactis* in humans, Steidler et al. replaced the chromosomal thymidylate synthase (*thyA*) with the Il10 transgene to generate a thymine auxotroph (Steidler 2002). The *thyA* gene is essential for the synthesis of thymine and thymidine and in the absence of these compounds the bacteria undergo thymineless death and lysis. Additionally, fragmentation of the genomic DNA occurs before lysis, reducing the risk of genetic transfer even further. Even in the unlikely event that *L. lactis* would acquire another lactococcal *thyA* gene by horizontal DNA transfer, recombination would lead to loss of the transgene. Viability of the *thyA* hIL10+ strain was reduced by several orders of magnitude in the absence of thymidine or thymine, and containment was validated in vivo in pigs (Steidler 2002). Currently, several

health authorities and biosafety committees have positively evaluated this containment system for environmentally released, genetically modified *L. lactis* (Rottiers et al. 2009). As mentioned above a small pilot trial with the *thyA* hIL10^{+} strain in patients with Crohn's disease showed that the containment strategy was highly effective (Braat et al. 2006).

FUTURE PERSPECTIVES

Although there are several examples of successful vaccination and protection using rLAB in rodent models, the next challenge will be to demonstrate their efficacy and advantages over injected vaccines in animals or humans. For vaccine delivery, it is evident that it is easier to obtain immune responses by i.n. administration and that even killed LAB are effective by this route. The use of killed LAB would have distinct advantages over live organisms with respect to the regulatory issues for the clinical use of genetically modified organisms, but the safety of i.n. immunization with a bacterial carrier needs to be addressed. Oral (i.g.) immunization remains a very attractive alternative, but frequent dosing over a period of several weeks may be required to achieve solid protection. However, recent targeting strategies for LAB show that it is possible to enhance the immunogenicity and efficacy of LAB vaccines.

The delivery of DNA vaccines using LAB is attractive given the potential to use mucosal routes of administration, but to date the immune responses have been much less potent than those reported for injected DNA vaccines. Efforts are underway to enhance DNA vaccine delivery by expressing pathogen invasins such as internalin A and FnBPA in LAB. The receptors for these invasins are typically expressed on the basolateral membrane of intestinal epithelial cells and thus appear to be only partially accessible from the lumen in selected parts of the epithelium, such as the tips of the villus. Indeed, recent studies indicate that only a small percentage of epithelial cells in vitro and in vivo take up targeted *L. lactis*. DNA vaccine antigens synthesized in epithelial cells could also contribute to the nature and potency of the immune response, but to effectively prime T cell responses LAB vaccines will have to be endocytosed by dendritic cells or other antigen-presenting cells that can effectively present the expressed antigen. The successful expression of LLO in *L. lactis* and the ability of the strain to induce CD8+ T cell responses indicate potential for combining it with cell-targeting strategies to facilitate release of the DNA vaccine vector into the cytoplasm.

Several research publications and clinical reports demonstrate the considerable potential to use genetically modified LAB to deliver therapeutic peptides and proteins to the mucosa. A containment system for the genetically modified *L. lactis* based on replacement of lactococcal *thyA* gene with the Il-10 transgene has been positively evaluated by several health authorities and biosafety committees and should pave the way for other LAB delivery applications in the future. Most research has focused on the medical need for new therapeutic approaches for IBD, but new medical targets, such as oral mucositis, are on the horizon (Rottiers et al. 2009). A promising recent development has been the finding that oral administration of *L. lactis* can be used to induce antigen-specific OT to a secreted recombinant antigen. LAB delivery induces much more efficient responses than with purified antigen, thus avoiding the need for purification of large quantities of antigen. This approach holds promise for new therapeutic interventions in allergies and antigen-induced autoimmune diseases.

DISCLOSURE STATEMENT

The author is a named inventor on patents relating to the use of recombinant lactic acid bacteria as delivery vehicles and has financial interests in a biotechnology company.

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