Evaluation of Oral Immunization with Recombinant Avian Influenza Virus HA1 Displayed on the Lactococcus lactis Surface and Combined with the Mucosal Adjuvant Cholera Toxin Subunit B

Han Lei, Zhina Sheng, Qian Ding, Jian Chen, Xiaohui Wei, Dominic Man-Kit Lam, and Yuhong Xu

College of Life Science and Food Engineering, Nanchang University, Nanchang 330013, People’s Republic of China; School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, People’s Republic of China; and Torsten Wiesel International Research Institute, Sichuan University, Chengdu, People’s Republic of China

Received 5 February 2011/Returned for modification 5 March 2011/Accepted 24 April 2011

The development of safe and efficient avian influenza vaccines for human and animal uses is essential for preventing virulent outbreaks and pandemics worldwide. In this study, we constructed a recombinant (pgs4-HA1 gene fusion) Lactococcus lactis strain that expresses and displays the avian influenza virus HA1 antigens on its surface. The vectors were administered by oral delivery with or without the addition of cholera toxin subunit B (CTB). The resulting immune responses were analyzed, and the mice were eventually challenged with lethal doses of H5N1 viruses. Significant titers of hemagglutinin (HA)-specific serum IgG and fecal IgA were detected in the group that also received CTB. Cellular immunities were also shown in both cell proliferation and gamma interferon (IFN-γ) enzyme-linked immunospot (ELISpot) assays. Most importantly, the mice that received the L. lactis pgs4-HA1 strain combined with CTB were completely protected from lethal challenge of the H5N1 virus. These findings support the further development of L. lactis-based avian influenza virus vaccines for human and animal uses.

The spreading of highly pathogenic avian influenza (HPAI) virus H5N1 remains a major global concern. In addition to the many outbreaks reported in various bird populations (13, 20) yearly all over the world, more than 516 human cases of H5N1 infection have also been reported (World Health Organization; http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_01_05/en/index.html). Since the viruses are going through constant genetic changes, one day they may acquire the ability to be transmitted among humans, leading to a human pandemic threat (30). Therefore, it is of prominent importance to implement safe and effective vaccination strategies to help contain viral spreading in animals and deter its development into a major threat to animal and human health.

In previous studies, there have been various constructs of H5 vaccines developed and tested (12). The most widely accepted strategy is to administer heat-inactivated H5N1 viruses produced in embryonated eggs. However, there have been many reports that this approach requires a large dose of the dead virus to elicit a protective immune response, and the serumconversion rates were suboptimum (6). In addition, the inefficient manufacturing procedure and inconvenient vaccination procedure (intramuscular injection) have prevented wider use of the vaccine constructs. Therefore, people are constant searching for more efficient and effective vaccine constructs.

Many studies have explored different vaccines focusing on the virus surface hemagglutinin (HA) antigen. Studies have shown that vaccine constructs containing recombinant HA protein (21), plasmid DNA encoding HA (7, 14, 36), or nonreplicative adenovirus-vectorized influenza vaccine (31, 32) could all induce protective immune responses in mice. However, they would all require intramuscular injection and even electroporation, which could still limit their application, especially to a large population of animals.

Recently, the use of Lactococcus lactis to present H5N1 virus antigen has been attempted (19). L. lactis is a Gram-positive lactic acid bacterium that is widely used for the production and preservation of fermented milk products, so it is generally regarded as safe (GRAS). In order to improve the immune efficiency of recombinant L. lactis, various approaches have been explored, such as oral dosages, route of administration, adjuvants, and antigen location (surface, cytoplasm, and extracellular) (18, 26); among these methods, the mucosal immune route (intranasal or oral) combined with the nontoxic B subunit of cholera toxin (CTB), which is a strong mucosal adjuvant, are regarded as the best way to improve immune efficiency (2, 15). After heterologous protein was displayed on the cell surface through the anchor domain, it showed a better immune outcome than proteins expressed intracellularly (17). The use of PgsA from Bacillus subtilis as a C-terminal anchor protein has been verified by Lee et al. (16).

In this study, we developed a construct expressing an HA1 antigen gene of H5N1 virus based on a Lactococcus lactis strain. L. lactis can be engineered to express various proteins, including bacterial and viral antigens, and can induce antigen-specific mucosal as well as systemic immune responses (11, 19, 22, 24, 25, 34). Most importantly, it can be safely administered orally, and we showed that immune protections against lethal
challenge of H5N1 virus could be achieved by optimizing the L. lactis-based construct and the mucosal formulation.

MATERIALS AND METHODS

Plasmid constructs and transformation. A, 1.136-bp fragment containing the pgsA gene was amplified from the chromosomal DNA of Bacillus subtilis by PCR using primers pgsA-F (5′-GAAGCTTGAAGAAGAAATCGGAGGTTGCTTCTA TG-3′) and pgsA-R (5′-CTCCTCTGGGCTATGTCCAGTATTTTAATTGGTCTC ACT-3′), in which the SpeI site is underlined. The 1.053-bp HA1 gene fragment encoding the HA1 protein that contained the sequence encoding the globular domain of HA from A/chicken/Henan/12/2004 (HSN1) (GenBank accession no. AY950232) was amplified using the following primer pairs with HindIII sites underlined (HA1-F, 5′-GGATCCGGAAGGAGATCAGATTGCTGGTAC G-3′; and HA1-R, 5′-CCGAGCTTTACCTCCTTTTTGTC-3′). Then the pgsA DNA fragment and HA1 DNA fragment were fused into pGEM-HE1 through a bridge PCR using primers pgsA-F and HA1-R, which contained SpeI and HindIII sites, respectively. For generation of a surface display plasmid, the resulting pgsA-HA1 DNA fragment was digested with SpeI-HindIII and inserted into similarly digested pNZ8110 under transcriptional control of the nisA promoter to generate pNZ8110-nisA-HA1 (Fig. 1A). The resulting plasmid was transformed into L. lactis NZ9000 by electroporation to create the L. lactis pgsA-HA1 strain. As a negative control, L. lactis NZ9000 was transformed with the pgsA gene to generate the L. lactis pgsA strain.

Nisin-controlled antigen expression. Recombinant L. lactis cells were cultured in M17 broth medium with 10 µg/ml chloramphenicol containing 0.5% (wt/vol) glucose (GM17) at 30°C overnight without agitation. Overnight cultures of recombiant L. lactis cells were diluted 1:25 in fresh GM17 medium containing 10 µg/ml chloramphenicol. When an optical density of 600 nm (OD600) of 0.3 to 0.4 was reached, nisin A was added at a final concentration of 10 ng/ml. Growth was continued for 3 h.

Western blot analysis. To confirm HA1 gene expression, L. lactis pgsA-HA1 and pgsA cells were harvested after induction and centrifuged at 5,000 × g for 8 min at 4°C. Cell pellets were washed twice with 20 mM Tris-HCl (pH 8.0), and the cell lysates and supernatants were analyzed by Western blotting. Briefly, each fraction was mixed with equal volumes of 2× sodium dodecyl sulfate (SDS) buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.01% bromophenol blue, and 10% β-mercaptoethanol). After boiling for 10 min, the cell lysates were electrophoresed on 10% acrylamide gel and transferred to nitrocellulose membrane. Protein was detected using polyclonal mouse anti-HA antibody followed by affinity-purified horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. The membrane was radiographed on X-film using the ECL Western blotting detection system (Pierce).

Flow cytometric analysis and immunofluorescence microscopy. To investigate whether HA1 protein was expressed on the surface of L. lactis, L. lactis pgsA-HA1 and pgsA cells were centrifuged at 5,000 × g for 10 min at 4°C and washed three times with sterile phosphate-buffered saline (PBS). The bacteria were then incubated with polyclonal mouse anti-HA antibody at 4°C overnight followed by biotinylated goat anti-mouse IgG and phycocerythrin (PE)-conjugated streptavidin. The stained cells were analyzed by flow cytometry (BD FACSCalibur).

For the immunofluorescence staining, the L. lactis pgsA-HA1 and pgsA cells were harvested after induction and incubated with polyclonal mouse anti-HA antibody at 4°C for 30 min following fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. The L. lactis pgsA strain was used as a negative control. Immunofluorescence analysis was examined using a fluorescence microscope (Olympus IX71, Japan).

Oral immunizations in mice. Six-week-old BALB/c female mice were purchased from SLC, Inc. (Shanghai, China), and housed in the Animal Center of the School of Pharmacy, Shanghai Jiao Tong University. The mice were kept under standard specific-pathogen-free (SPF) conditions and provided with free access to food and water during the experiments.

After overnight fasting, groups of 10 mice received, using an oral Zonde needle, 150 µl of 10^9 CFU of the L. lactis pgsA-HA1 or pgsA strain plus 1 mg CTB, which was purchased from Sigma as a recombinant protein. For comparison, other groups were administered with the same dose of the L. lactis pgsA-HA1 and pgsA strains. Immunization was performed daily via intragastric lavage on days 0 to 3, 8 to 11, and 24 to 28.

ELISA. Samples of sera (collected by retro-orbital puncture) and feces were collected 38 days after the final immunization. Sera were stored at −20°C until use. Fecal pellets (50 mg) were suspended in 0.3 ml sterile PBS. After centrifugation at 12,000 × g for 5 min, the supernatants were collected and tested for IgA by enzyme-linked immunosorbent assay (ELISA). IgG detection was performed by avidin-biotin system (ABS)-ELISA (37); the IgA antibody was assayed by indirect ELISA. Briefly, 96-well microplates were coated with recombinant influenza A virus [A/chicken/Henan/12/2004(H5N1)] HA protein overnight at 4°C. The wells were blocked with PBS–1% bovine serum albumin (BSA) and incubated for 2 h at room temperature. Two-fold serially diluted serum or fecal suspension (100 µl) was added for 1 h at 37°C. Bound antibody was detected using biotin-conjugated goat anti-mouse IgG, alkaline phosphatase-conjugated streptavidin (R&D Systems), or HRP-conjugated goat anti-mouse IgA (Sigma). The mean antibody titer was expressed as the reciprocal of the serial serum dilution that reached the cutoff value plus 2 standard deviations (SD).

IFN-γ ELISPot assay. Gamma interferon (IFN-γ)-producing cells were quantified 1 week after the final immunization using an ELISPot kit (R&D Systems) according to the manufacturer’s instructions. Briefly, to a mouse IFN-γ microplate was added 100 µl/well of 1 × 10^6 splenocytes and 10 µg/ml of HA-specific peptide (ISVGTSTLNQRLVP), which were used as stimuli for 48 h in a humidified 37°C CO2 incubator. Control wells contained unstimulated cells. After incubation, each well was washed, and the plates were incubated sequentially with biotinylated anti-mouse IFN-γ antibody, alkaline phosphatase-conjugated streptavidin, and the substrate solution to reveal the spots. The numbers of spots were counted with a dissection microscope. All assays were performed in triplicate.

H5N1 virus challenge experiments. Influenza A virus [A/chicken/Henan/12/2004(H5N1)] was used for the virus challenge experiments. Two weeks after the final immunization, mice (n = 5 mice per group) were challenged intranasally with 5 50% lethal doses (LD₅₀) of HSN1 virus in a volume of 20 µl. Mice were monitored for disease signs and death for 14 days after infection. All experiments were performed under high-containment enhanced biosafety level 3 (BSL3) conditions.

Statistical analysis. Statistical analyses were performed using Student’s t test for data, and P values of <0.05 were considered statistically significant.

RESULTS

Construction of recombinant L. lactis pgsA-HA1 and HA1 protein expression in vitro. The pgsA and HA1 DNA fragments were fused in a bridge PCR system to generate the pgsA-HA1 gene. After digestion, the pgsA-HA1 gene was transcriptionally fused with pNZ8110 to yield pNZ8110-pgsA-HA1 (Fig. 1A). The resulting pNZ8110-pgsA-HA1 plasmid was transformed into the L. lactis NZ9000 strain. Purified plasmids from recombinant L. lactis pgsA-HA1 and pgsA strains were detected by agarose gel electrophoresis (Fig. 1B). As shown in lane 2, the pgsA-HA1 fragment (2,189 bp) was successfully digested. Further Western blot analysis of cell lysates and supernatants showed HA1 was located on the surface of the L. lactis pgsA-HA1 strain or intracellularly (Fig. 1C). The band of 82 kDa most likely represents the HA1 protein (38 kDa) and the PgsA surface display motif (44 kDa).

To determine whether this HA1 protein was expressed on the surface of L. lactis via a cell wall anchor, fluorescence-activated cell sorter (FACS) analysis revealed that a significant increase in fluorescence intensity was obtained in the L. lactis pgsA-HA1 strain (Fig. 1D). Immunofluorescence further showed that cells harboring the L. lactis pgsA-HA1 strain immunostained positive for HA1, but control cells harboring the L. lactis pgsA strain did not (Fig. 1E). These data indicated our constructed recombinant L. lactis strain could make the HA1 protein effectively displayed on the surface of the cell wall.

Immune responses induced by oral vaccination with L. lactis pgsA-HA1 (CTB) in mice. A total of 150 µl 10^10 CFU of the L. lactis pgsA-HA1 or pgsA strain with pgsA (CTB) or without CTB was orally administered to BALB/c mice (SPF grade). The effects of vaccination on the production of HA-specific serum IgG and fecal IgA were examined 10 days after the final immunization. As shown in Fig. 2A, when mice were orally administered the L. lactis pgsA-HA1 (CTB) strain, the serum
IgG antibody titer obtained showed a significant increase compared with those of the other groups. Fecal IgA was also examined and was significantly produced after immunization with the *L. lactis* *pgsA*-HA1 (CTB) strain (Fig. 2B). These data demonstrated that administration to mice of the *L. lactis* *pgsA*-HA1 (CTB) strain resulted in significant enhancements of HA-specific IgG and mucosal IgA compared with controls administered PBS or the *L. lactis* *pgsA*, *pgsA*-HA1, and *pgsA* (CTB) strains.

*L. lactis* *pgsA*-HA1 (CTB) strain immunization stimulates HA-specific cellular immune response. To determine whether the *L. lactis* *pgsA*-HA1 (CTB) strain could induce cellular immunity, lymphoproliferation and IFN-γ ELISpot assays were performed. The splenocytes were stimulated with 2 μg/ml
recombinant HA protein, and the proliferation levels were reflected by absorbance at 450 nm on a spectrophotometer. Compared to the results from mice immunized with PBS or the *L. lactis* pgsA, pgsA-HA1, or pgsA (CTB) strains, significant enhancement was observed by the IFN-γ ELISPOT assay: the number of HA-specific IFN-γ-secreting cells in *L. lactis* pgsA-HA1 (CTB)-treated mice was significantly greater than those in the other groups (*P* < 0.05) (Fig. 3B). These results suggested that oral administration of the *L. lactis* pgsA-HA1 (CTB) strain could effectively induce HA-specific cell mediated responses (cell-mediated immunity [CMI]).

**Protection against lethal H5N1 virus challenge.** To test whether vaccinated mice could stand against H5N1 virus challenge, all mice (5 mice in each group) were infected by intranasal drip with a lethal dose of 20 μl of 5 LD₅₀. After H5N1 virus infection, the survival rate was observed for 14 days. The result in percent survival indicated mice immunized with the *L. lactis* pgsA-HA1 (CTB) strain obtained 100% protection. In contrast, other groups were killed within 10 days (Fig. 4). These data demonstrated that the *L. lactis* pgsA-HA1 (CTB) strain was an effective vaccine for providing complete protection against H5N1 virus challenge.

**DISCUSSION**

This study demonstrated that oral administration of recombinant *L. lactis* strains with surface-displayed antigens combined with mucosal adjuvant CTB could induce sufficient HA-specific mucosal and systemic immune responses for protection against H5N1 virus lethal challenge in BALB/c mice.

Mucosal surfaces are the first line of defense for effective elimination of many pathogens. For the prevention of highly pathogenic avian influenza virus H5N1 infection, it is considered favorable to develop sufficient mucosal immunity at the first line of defense (35). Vaccination by the mucosal route is needed to generate a mucosal immune response. Prabakaran et al. constructed a recombinant baculovirus with surface-displayed HA protein and showed that it was more efficacious than inactivated H5N1 influenza vaccine by intranasal administration. They showed that the vector when combined with CTB as an adjuvant could achieve up to 100% protection from homogenous H5N1 virus challenge in mice (23). Saluja et al. showed influenza virus subunit vaccine formulated with Gram-positive enhancer matrix (GEM) particles as an adjuvant could produce effective protection, and they concluded that GEM from *L. lactis* was a safe adjuvant (27). However, the intranasal route of administration has limitations in dosing volume and mucosal exposure, and the delivery efficiencies may be highly variable.

The oral administration route is considered much more convenient in comparison, and the antigens can also have access to a larger mucosal area for a prolonged duration. However, proteins delivered orally are easily degraded and have very low immunogenicity (3), so it is essential to develop specific vaccine delivery systems and adjuvants to facilitate the antigen’s interaction with the host’s immune system. *L. lactis* is a non-pathogenic, noninvasive, and noncommensal food-grade bacterium that has been considered an ideal mucosal delivery vector (33). *L. lactis*-derived vectors are widely used and have a superior safety profile (33) compared to other bacterially based live vectors, such as *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (1), *Listeria monocytogenes* (29), *Salmonella* (9), and *Shigella* (28). However, it may have less immunogenicity than more virulent vectors. In order to improve
the immunogenicity of recombinant \textit{L. lactis}, the use of an effective adjuvant or modifications of recombinant vectors would be necessary.

Previously, we reported the development of a recombinant \textit{L. lactis} strain for oral immunization in enteric minicapsules (19). We used the nisin-controlled expression (NICE) system to construct \textit{L. lactis} vectors that express and secret the HA antigen. In this study, we further modified the expression system to display the HA1 antigen protein on the surface of \textit{L. lactis} through the PgsA anchor motif. We believe such modification can further improve the antigen’s stability orally and at the same time facilitate antigen presentation because bacteria cell wall anchoring proteins were found to be more resistant to proteolysis and easily taken up by the epithelial M cell of the small intestine (33).

Furthermore, for orally administered vaccines, it is also considered essential to incorporate special adjuvants to boost interaction with the mucosal immune system. Various synthetic vectors such as microspheres, liposomes, nanoparticles, and immunostimulating complexes (ISCOMs) have been tested and showed improved immune responses, but the efficacies were not optimal (5). Certain bacterial toxins such as the cholera toxin (CT) and \textit{Escherichia coli} heat-labile enterotoxin (LT) were considered more immunogenic (4). Although these toxins are toxic in general, previous studies had shown that the B subunit may be a benign functional unit involved only in the recognition and binding to ganglioside GM1 cell receptors. Recombinant CTBs or LT subunits B (LTBs) had been used successfully in several vaccination studies as adjuvants with oral administration (4, 8). They can stimulate upregulation of B7-2 expression on antigen-presenting cells (APCs) and promote Th2 immune responses (10). In this study, we confirmed the immune-stimulating effect of CTB. The CTB proteins were simply mixed with the HA-expressing \textit{L. lactis} vectors and administered orally. Both the resulting IgG and IgA responses were higher, and the cell-mediated response was also improved compared to the data obtained with the \textit{L. lactis} vectors alone (Fig. 2 and Fig. 3). We think the immune-adjuvanting effect of CTB in our study is critical to generate
protective immunity against H5N1 virus challenge. Experiments with heterologous cross-protection against various divergent strains are ongoing.

In summary, we constructed a recombinant L. lactis strain in which the HAI antigen was displayed on the surface of the cell wall. Oral administration of this mucosal vaccine combined CTB-induced strong HA-specific humoral and mucosal immune responses that significantly stand against challenge with a lethal dose of H5N1 virus. These findings indicate that a recombinant L. lactis strain can be used as a safe, effective, and inexpensive mucosal vaccine delivery vector.

ACKNOWLEDGMENT

This work was supported by Natural Science Foundation of China grant no. 30825045 to Y. Xu.

REFERENCES

1. Aldovini, A., and R. A. Young. 1991. Humoral and cell-mediated immune response to live recombinant BCG-HIV vaccines. Nature 351:479–482.
2. Asahi-Ozaki, Y., et al. 2008. Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect. 8:2706–2714.
3. Bahey-El-Din, M., P. G. Casey, and B. T. Griffin. 2008. Lactococcus lactis expressing listeriolysin O (LLO) provides protection and specific CDS+ T cells against Listeria monocytogenes in the murine infection model. Vaccine 26:5304–5314.
4. Bermúdez-Humaran, L. G. 2009. Lactococcus lactis as a live vector for mucosal delivery of therapeutic proteins. Hum. Vaccin. 504:264–267.
5. Bermúdez-Humaran, L. G., G. Corblier, and P. Langella. 2004. Recent advances in the use of Lactococcus lactis as live recombinant vector for the development of new safe mucosal vaccines. Recent Dev. Microbiol. 8:147–160.
6. Carter, N. J., and G. L. Plosker. 2008. Preparimend influenza vaccine H5N1 (split virion, inactivated, adjuvanted) [Prepandrix]: a review of its use as an active immunization against influenza A subtype H5N1 virus. BioDrugs 22:279–292.
7. Chen, M. W., et al. 2008. A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. Proc. Natl. Acad. Sci. U. S. A. 105:13538–13543.
8. Cong, Y., C. T. Weaver, and C. O. Elson. 1997. The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. J. Immunol. 159:5301–5308.
9. Darji, A., et al. 1997. Oral somatic transgene vaccination using attenuated S. typhimurium. Cell 91:765–775.
10. Elson, C. O., and W. Ealding. 1985. Genetic control of the murine immune response to cholera toxin. Eur. J. Immunol. 15:930–932.
11. Gilbert, C., K. Robinson, R. W. Le Page, and J. M. Wells. 2000. Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in Lactococcus lactis. Infect. Immun. 68:3251–3260.
12. Horimoto, T., and Y. Kawaoaka. 2006. Strategies for developing vaccines against H5N1 influenza A virus. Trends Mol. Med. 12:506–514.
13. Kou, Z., et al. 2009. The survey of H5N1 flu virus in wild birds in 14 provinces of China from 2004 to 2007. PLoS One 4:e6926.
14. Lafar, P. A., et al. 2008. Plasmid DNA-based vaccines protect mice and ferrets against lethal challenge with A/Vietnam/1203/04 (H5N1) influenza virus. J. Infect. Dis. 197:1643–1652.
15. Langridge, W., B. Denes, and I. Fodor. 2010. Cholera toxin B subunit modulator of mucosal vaccines for infectious and autoimmune diseases. Curr. Opin. Invest. Drugs 11:919–928.
16. Lee, J. S., et al. 2006. Mucosal immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on Lactobacillus casei induces neutralizing antibody in mice. J. Virol. 80:4079–4087.
17. Lee, J. S., K. S. Shin, J. G. Pan, and C. J. Kim. 2000. Surface-displayed viral antigen on Salmonella carrier vaccine. Nat. Biotechnol. 18:645–648.
18. Lee, M. H., Y. Rousseau, M. Wilks, and S. Tabadghali. 2001. Expression of Helicobacter pylori urease subunit B gene in Lactococcus lactis MG1363 and its use as a vaccine delivery system against H. pylori infection in mice. Vaccine 19:3927–3935.
19. Lei, H., Y. Xu, J. Chen, X. Wei, and D. M. Lam. 2010. Immunoprotection against influenza H5N1 virus by oral administration of enteric-coated recombinant Lactococcus lactis mini-capsules. Virology 407:319–324.
20. Li, K. S., et al. 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430:209–213.
21. Lin, J., et al. 2006. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 368:991–997.
22. Medina, M., et al. 2008. Nasal immunization with Lactococcus lactis expressing the pneumococcal protective protein A induces protective immunity in mice. Infect. Immun. 76:2696–2705.
23. Prabakaran, M., et al. 2008. Protective immunity influenza H5N1 virus challenge in mice by intranasal co-administration of baculovirus surface-displayed HA and recombinant CTB as an adjuvant. Virology 380:412–420.
24. Quigley, B. R., et al. 2010. A foreign protein incorporated on the tip of T3 pili in Lactococcus lactis elicits systemic and mucosal immunity. Infect. Immun. 78:1294–1303.
25. Robinson, K., L. M. Chamberlain, K. M. Schofield, J. M. Wells, and R. W. Le Page. 1997. Oral vaccination of mice against tetanus with recombinant Lactococcus lactis. Nat. Biotechnol. 15:653–657.
26. Robinson, K., et al. 2004. Mucosal and cellular immune responses elicited by recombinant Lactococcus lactis strains expressing tetanus toxin fragment C. Infect. Immun. 72:2753–2761.
27. Saluja, V., et al. 2010. Intranasal delivery of influenza subunit vaccine formulated with GEM particles as an adjuvant. AAPS J. 12:109–116.
28. Shata, M. T., and D. M. Hone. 2001. Vaccination with a Shigella DNA vaccine vector induces antigen-specific CDS+ T cells and antiviral protective immunity. J. Virol. 75:9665–9670.
29. Shen, H., et al. 1998. Compartimentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity. Cell 92:515–545.
30. Smith, G. J., et al. 2006. Emergence and predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. U. S. A. 103:16936–16941.
31. Steitz, J., et al. 2010. Assessment of route of administration and dose escalation for an adenovirus-based influenza A (H5N1) vaccine in chickens. Clin. Vaccine Immunol. 17:1467–1472.
32. Van Kampen, K. R., et al. 2005. Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. Vaccine 23:1029–1036.
33. Wells, J. M., and A. Mercenier. 2008. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat. Rev. Microbiol. 6:349–362.
34. Xin, K. Q., et al. 2003. Immunogenicity and protective efficacy of orally administered recombinant Lactococcus lactis expressing surface-bound H1N1 Env. Blood 102:223–228.
35. Ye, J., et al. 2010. Intranasal delivery of an IgA monoclonal antibody effective against sublethal H5N1 influenza virus infection in mice. Clin. Vaccine Immunol. 17:1363–1370.
36. Zheng, L., et al. 2009. A single immunization with HA DNA vaccine by electroporation induces early protection against H5N1 avian influenza virus challenge in mice. BMC Infect. Dis. 9:17.
37. Zhou, Y., et al. 2008. Electroporation at low voltages enables DNA vaccine to provide protection against a lethal H5N1 avian influenza virus challenge in mice. Intervirology 51:241–246.