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Avian reovirus sigma C enhances the mucosal and systemic immune responses elicited by antigen-conjugated lactic acid bacteria

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ABSTRACT

Mucosal surfaces are common sites of pathogen colonization/entry. Effective mucosal immunity by vaccination should provide protection at this primary infection site. Our aim was to develop a new vaccination strategy that elicits a mucosal immune response. A new strain of *Enterococcus faecium*, a non pathogenic lactic acid bacteria (LAB) with strong cell adhesion ability, was identified and used as a vaccine vector to deliver two model antigens. Specifically, sigma (σ) C protein of avian reovirus (ARV), a functional homolog of mammalian reovirus σ1 protein and responsible for M-cell targeting, was administered together with a subfragment of the spike protein of infectious bronchitis virus (IBV). Next, the effect of immunization route on the immune response was assessed by delivering the antigens via the LAB strain. Intranasal (IN) immunization induced stronger humoral responses than intragastic (IG) immunization. IN immunization produced antigen specific IgA both systemically and in the lungs. A higher IgA titer was induced by the LAB with ARV σC protein attached. Moreover, the serum of mice immunized with LAB displaying divalent antigens had much stronger immune reactivity against ARV σC protein compared to IBV-S1. Our results indicate that ARV σC protein delivered by LAB via the IN route elicits strong mucosal immunity. A needle-free delivery approach is a convenient and cost effective method of vaccine administration, especially for respiratory infections in economic animals. Furthermore, ARV σC, a strong immunogen of ARV, may be able to serve as an immunoenhancer for other vaccines, especially avian vaccines.

1. Introduction

Mucous membranes constitute the largest interface between the body and the external environment and serve as a physical barrier that prevents the entry and dissemination of infectious pathogens. In addition, it contains immunocompetent cells that are required for the generation of antigen-specific mucosal immunity, the crucial immunological defense against invading microorganisms.

Microfold (M) cells, located in follicle-associated epithelium (FAE), transport the antigens, which include both soluble proteins and infectious agents, from the intestinal and respiratory tract lumen to the mucosa-associated lymphoid tissues (MALT), namely Peyer's patches, and nasopharynx-associated lymphoid tissue (NALT), respectively [1,2]. In the MALT, dendritic cells process and present antigens to T cells that in turn preferentially induce the production of secretory IgA (SIgA) at mucosal surface [3]. This IgA effectively neutralizes and prevents attachment and internalization of pathogens into host cells. Hence, the elicitation of mucosal immunity is critical when developing vaccines against infectious pathogens that are transmitted through mucosal sites.

Vaccine strategies that target mucosal immunity have many advantages; these include the capacity to elicit both systemic and mucosal immune responses together with improved accessibility, safety, and cost-effectiveness because the delivery is needle-free [4]. Several lines of evidence have indicated that induction of adequate mucosal protective immune response requires the aid of adjuvants. At present, the most effective mucosal adjuvants are Cholera toxin and *Escherichia coli* lymphotoxin [5]; however, considering their potential toxicity, these bacterial toxins are not acceptable for clinic use [6]. Lactic acid bacteria (LAB), which are generally classified as GRAS (Generally Recognized As Safe)

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organisms, are traditionally used in the food industry. Recently, the potential application of LAB as vehicles for the expression and delivery of model antigens to mucosal surfaces has been extensively investigated [7–10]. Bermudez-Humaran et al., showed that mice intranasally administered with *Lactococci lactis* (LL), having the E7 antigen of human papillomavirus type 16 (LL-E7) anchored to the cells together with the secretory form of interleukine-12 (LL-IL-12), induced an E7-specific immune response and provided therapeutic effects [7,8]. Cortes-Perez has further demonstrated that simultaneous immunization of the LL-E7 and LL-IL-12 strains via the intranasal route is able to elicit higher specific immune responses and more potent anti-tumor effects than that via the intragastric route [11]. In addition, Mohamadzadeh et al., reported that co-culture with *Lactobacillus* promotes DC maturation and up-regulates expression of IL-12 and IL-18, but not IL-10. These results suggest that LAB regulate T cell responses and target them toward the T helper 1 pathway [12]. Hence, LAB strains act not only as a delivery vehicle, but also as an intrinsic adjuvant during vaccination. Nevertheless, the effect of LAB on immunomodulation may vary dependent with the LAB species or strain [13]. Thus the routes of immunization and differences in the intrinsic adjuvanticity of LAB strains need to be considered when planning an immunization strategy. In addition to *Lactobacillus*, the probiotic effect of *Enterococcus faecium* (*E. faecium*) has been demonstrated [14–16]. It is worthy of noting that unlike most of LAB, the *Enterococcus* genus is not considered as "GRAS". For safety concern and to avoid antibiotic resistant gene transmission, a case-by-case evaluation of pathogenicity and antibiotics resistance profiles is required [17].

Avian infectious bronchitis virus (IBV) is a member of group 3 of the coronaviruses and is a highly contagious disease of chickens [18,19]. The spike glycoprotein, which is involved in cell attachment, is an immunodominant protein that carries epitopes that produce virus-neutralizing antibodies [20]. Previously, Lee et al., found that intranasal and oral immunization of mice with *Lactobacillus casei* displaying the spike protein of SARS-coronavirus on its surface elicited protective systemic and mucosal immune responses against SARS pseudovirus [9]. Based on this finding, a previously defined antigenic epitope of the Spike protein of the IBV Taiwan local strain (TW1) was selected as one of the model antigens. This was used for the induction of the antigen-specific antibodies and evaluated in current study.

Avian reoviruses (ARV) are classified into the orthoreovirus genus of the family *Reoviridae*. Sigma (σ) C protein, a component of the outer capsid layer of the ARV, is responsible for attachment to the host cell membrane [21] and can induce high levels of type-specificity neutralization antibodies [22]. Structure-based sequence alignment of the ARV sigma C and mammalian reovirus (MRV) type 3 sigma 1 have indicated the presence of heptad repeats and a triple alpha-helical coiled-coil structure in N-terminal region [23]. In addition, crystallographic studies found that the carboxy-terminal globular domain of ARV sigma C has a similar overall topology to that of MRV type 3 sigma 1. It has been shown that MRV sigma 1 protein recognizes the receptor of M cells (α-2-3 linked sialic acid) that facilitates penetration of antigens into intestinal Peyer's patches [24]. Mishra et al. further reported that encapsulation of hepatitis B surface antigen by nanocarriers or liposome with M-cell targeting ligands (lectins, including *Ulex europaeus agglutinin* 1; UEA-1) promotes intestinal uptake by M-cells, enhances antibody production and increases cellular immune responses [25,26]. As immunomodulation of ARV sigma C has not yet been reported, we investigated whether ARV sigma C protein has a similar effect as MRV sigma 1 on the enhancement of mucosal immunization.

In this study, LysM of AcmA', the autolysin of *Lactococcus lactis*, which can exogenously bind to the peptidoglycan of LAB [27], was chosen as the anchor molecule for docking each of the desired antigenic protein onto the LAB surface. The two model antigens, IBV-spike and ARV-sigma C, were expressed as a AcmA' tag fused at the C-terminal end. The display of recombinant proteins on the LAB surface was confirmed and the immune response induced by the antigen-displaying LAB was evaluated using an animal model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The lactic acid bacteria (LAB), namely three *Enterococcus faecium* (*E. faecium*) strains, were obtained, one from the Food Industry Research and Development Institute, Taiwan, (ATCC-6057) and two being isolated from pig intestine flora (58a-1, 63b-2). These were cultured on *Lactobacilli* MRS broth (Difco™ Detroit, Mich., USA) at 37 °C without shaking. To quantify the bacteria, the *E. faecium* was grown to logarithmic phase, collected by centrifugation, and then washed three times with phosphate buffer saline (PBS). The initial concentration of the bacteria was determined by spectrophotometry at OD 600 nm. The number of bacteria is each sample was verified by pour-plate assay using Ragosa agar plate (Difco™ Detroit, Mich., USA) and ten-fold serial dilution. Finally, the LAB were diluted in 1×PBS at a concentration of 10^{10} colony forming units (CFU)/ml. *Escherichia coli* (*E. coli*) Top10 (Invitrogen) and BL21 (DE3) (Novagen), which were used for plasmid amplification and protein expression, respectively, and were grown in LB medium with shaking at 37 °C.

2.2. Cell culture

The intestine cell line Int 407 (ATCC: CCL-6) was cultured using BME (Basal medium Eagle in Earle's BSS) with 10% fetal bovine serum (FBS), penicillin 100 U/ml, and streptomycin 100 μg/ml.

2.3. Animals

Female BALB/c mice, 5–8 weeks old, were purchased from the National Laboratory Animal Center (Taiwan). All experiments were performed in accordance with the University Guidelines.

2.4. Plasmid construction

To generate the recombinant proteins for immunization, plasmid expressing antigenic protein fused with the AcmA' protein, which serves as an anchoring protein onto Gram positive bacteria such as lactic acid bacteria (LAB) [28], were constructed in two steps. Initially, AcmA′ fragment was obtained by PCR from the DNA of *L .lactis* (cremoris SK11) using the primer sets (AcmA′-F: CGACAAGCTTGCACGGACGGAGCTTCTTC, and AcmA′-R: GGTGCTCGAGTGAACCACCTGAATTTGTA), which were designed based on sequences published in GenBank (accession number: AF036720). The thermal cycling conditions were: 94 °C (3 min) followed by 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 45 s), and extension (72 °C, 1 min), and finished with a final extension (72 °C, 3 min). The PCR product had the expected size of 261 base pairs (bp) and was then digested with *Hin*d III and *Xho* I restriction enzymes for subcloning into the prokaryotic expression vector pET24a. The resulting plasmid was named AcmA'-pET24a.

Based on a previous report [29], an epitope consisting of amino acids residues 235–302, which are highly conserved in S1 subunit of infectious bronchitis virus (IBV) spike (S) protein and show antibody neutralizing activity, was chosen for use in this study. Primers were designed to amplify the S1 subfragment (residues 235–302) of the IBV TW1 strain (GenBank accession number: DQ646405) by PCR. The primer sequences used were: IBV-S1-F: TGCTAGCTAGCCAATATAATACTGG and IBV-S1-R: AAAATGTCGACAAGCTTTCTGAGCTG. A plasmid IBV-S-pTA